

UNIVERSITY OF NAPLES FEDERICO II



DOCTORAL SCHOOL OF "AGROBIOLOGIA E
AGROCHIMICA" (XXVIII CYCLE)

*Biochar as a new soil amendment to
promote plant growth and disease control*

Dott.ssa Ippolito Francesca

Tutor:
Prof. F. Scala

Coordinator:
Prof. D. Carputo

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***To freedom, first step of
research***

***Doing what you like is
freedom***

***Liking what you do is
happiness***

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Chapter 1

Introduction and aim

1. INTRODUCTION AND AIM

Recently, several methodologies are being developed for a more sustainable agriculture with a low environmental impact. Soil microbial communities play an important role in soil-plant relationships which may be related to root exudates and nutrients present in the rhizosphere region. The microflora in the rhizosphere is composed of different organisms (bacteria, fungi, oomycete, yeasts, algae and protozoa) that are involved in the degradation of organic matter, take part in the trophic processes in soil, suppress plant diseases and promote plant growth.

In particular, the use of plant growth promoting rhizobacteria (PGPR) increases root development and explore parts of the not colonized rhizosphere rich in nutrients. The use of biocontrol agents; both of bacterial (*Pseudomonas*, *Bacillus*, *Agrobacterium* etc.) and/or fungal (*Trichoderma*) origin reduces the competitiveness to plants, protects from soilborne pathogens by limiting or completely eliminating the incidence of diseases.

The use of natural organic amendments (compost and biochar) instead of synthetic chemical products (fertilizers and pesticides) improves the physicochemical characteristics of the soil and promotes root development, plant growth and productivity of crops without altering the composition of the microbial soil community.

Use of biochar dates back to the discovery of soil with high fertility in Central Amazon, called "Terra Preta do Indios", in contrast to adjacent poor soil, incapable to sustain the growth of crops for long time. This drew the attention of the scientific community (O'Neil, 2006). Numerous studies have demonstrated that these soils have a high content of nutrients, stable organic material and high capability of cation exchange (Galser et al., 2001; Liang et al., 2006; Steiner et al., 2004). There were many hypotheses to explain the origin of such fertility, leading to the conclusion that the high abundance of charcoal (biochar) was the ecological factor of major importance in this context. Biochar is defined as the product of thermal degradation of organic materials in absence of oxygen (pyrolysis). In recent years, several researchers have studied the effects of adding biochar to the soil in order to determine the factors and interactions that contribute to the success of Terra Preta (Lehmann et al., 2003).

Several tests in both pots and open field have shown that the addition of different biochars to the soil can improve the productivity of crops (Asai et al., 2009; Graber et al., 2010; Major et al., 2010). Addition of biochar to the soil leads to an increase of pH, capability of cation exchange, water and nutrient retention and improve the physical characteristics of

the soil (Atkison et al., 2010). However, few studies dealt with the effect of biochar on soilborne and airborne pathogens. For example, Harel et al. (2012) have shown the ability of biochar to induce a systemic response of strawberry to foliar pathogens *Colletotrichum acutatum* and *Botrytis cinerea*.

The aim of the present thesis was to investigate the relationship between the telluric microorganisms (fungal and bacterial pathogens and saprophytes) and the biochar in order to assess the potential of these materials for biological control. This thesis includes a review concerning disease suppressiveness by the new organic amendment biochar, with special focus on the limited studies present in the literature and the different mechanisms proposed to explain biochar disease suppression (chapter 1). The plant and microbial bioassays carried out on 48 litter types heated at five different temperatures (100, 200, 300, 400 and 500°C) were presented in chapter 2. Moreover, the relationship between litter biochemical quality defined by ^{13}C NMR spectroscopy and growth of target species were assessed. Finally, chapter 3 presented the interactions of biochar with plant growth and microbial activity also related to the ^{13}C NMR analyses and their physical and biochemical characteristics.

Chapter 2

A “black” future for plant pathology?
Biochar as a new soil amendment for
controlling plant diseases

2. A “BLACK” FUTURE FOR PLANT PATHOLOGY? BIOCHAR AS A NEW SOIL AMENDMENT FOR CONTROLLING PLANT DISEASES

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2.1 SUMMARY

The utilization of organic amendments has been proposed to decrease the incidence of plant diseases caused by soilborne pathogens. In this work we reviewed reports concerning disease suppressiveness by the new organic amendment biochar. A total of 12 papers were included in this analysis with 18 experimental case studies. The effect of biochar amendment was suppressive in 89% and non-significant in the remaining 11%, with few studies reporting a significant increase of disease incidence. Biochar can be effective against both airborne (e.g. *Botrytis cinerea*, different species of powdery mildew) and soilborne pathogens (e.g. *Fusarium* spp., *Phytophthora* spp., *Rhizoctonia solani*). Five different mechanisms have been proposed to explain biochar disease suppression: (i) induction of systemic resistance in host plants; (ii) enhanced abundance and activities of beneficial microbes, including mycorrhizal fungi; (iii) modification of soil quality in terms of nutrient availability and abiotic conditions such as liming effect; (iv) direct fungitoxic effect of biochar; (v) sorption of allelopathic, phytotoxic compounds that can directly harm plant roots and thus promote pathogen attacks. Potential side-effects of biochar have been reported, such as the possibility of absorbing agrochemicals such as herbicides, insecticides and fungicides, thus reducing their efficacy. Results from this review demonstrate that biochar amendments have great potential but, until now, not enough studies are available for a widespread adoption of biochar as a soil amendment in today's agricultural systems. More investigations on the mechanisms underlying biochar disease suppression, as well as long-term field experiments, are needed to make biochar a safe, effective and affordable tool for the control of plant pathogens.

Key words: Allelopathy, *Botrytis cinerea*, compost, diseases suppression, *Fusarium* spp., phytotoxicity, *Rhizoctonia solani*.

2.2 WHAT IS BIOCHAR?

Biochar is heterogeneous material generated through pyrolysis, i.e. a thermal process carried out at temperatures ranging from 200°C to 900°C and under limited oxygen availability, of a wide range of organic materials including crop residues (Yuan *et al.*, 2011), wood (Spokas and Reicosky, 2009), municipal waste (Mitchell *et al.*, 2013), sewage sludge (Méndez *et al.*, 2012), manure (Uzoma *et al.*, 2011), and also animal bones (Vassilev *et al.*, 2013). The International Biochar Initiative defined biochar as “a solid material obtained from the thermo-chemical conversion of biomass in an oxygen limited environment” (IBI, 2012). Biochar is basically distinguished from charcoal by its final end use: agriculture and environmental management in the first case, fuel and energy in the latter (Lehman and Joseph, 2009).

The positive effects of biochar on soil quality and crop performances have been known since ancient times: Pre-Columbian populations of Amazonia developed the so called “*terra preta*” or “*dark earth*” soils by repeating cycles of fire and cultivation, i.e. the slash-and-char cultivation system (Steiner *et al.*, 2004). In this way nutrient poor and highly weathered acidic soils were transformed into a fertile substrate capable of sustaining indigenous populations (Tollefson, 2013). However, this ancient empirical evidence found scientific support only in the last ten years, when a burst in research effort on this topic took place (Fig. 1A). Three main applications prompt scientific research: mitigation of climatic change (Lehmann, 2007), efficient and cost effective waste management (Navia and Crowley, 2010), and the use of biochar as amendment to improve soil quality and sustain crop yield (Sohi *et al.*, 2010).

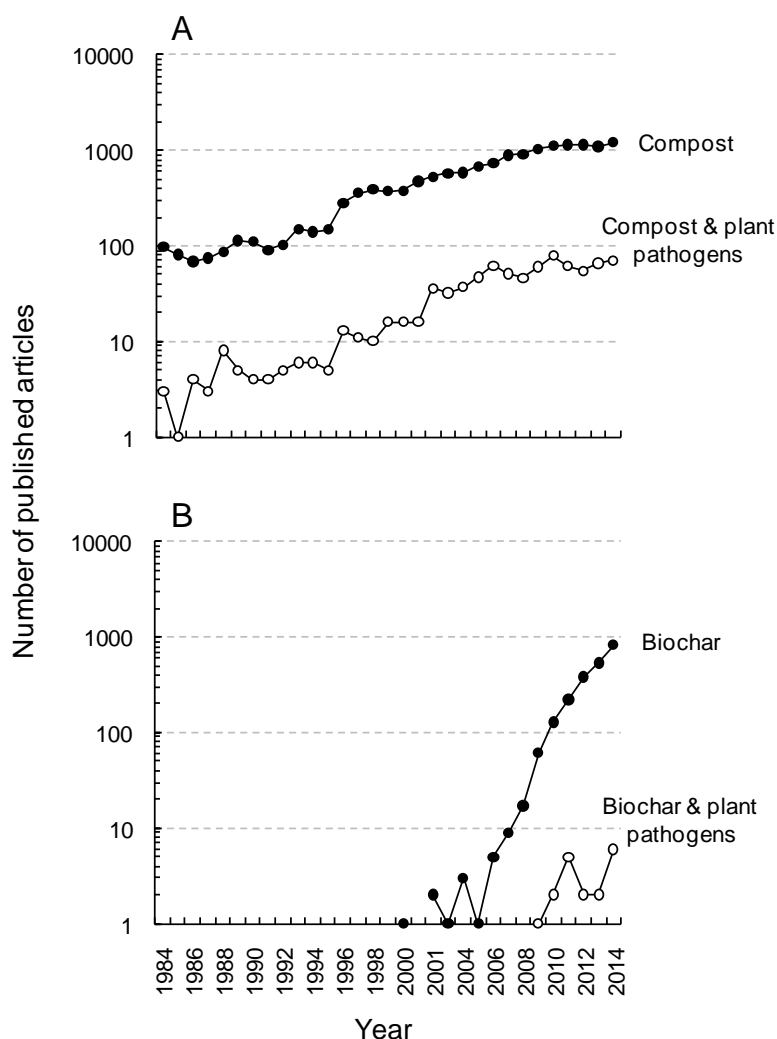
The use of biochar as soil amendment has been proposed to mitigate global climatic change by reducing green-house gas emission. Biochar has shown to be very resistant to microbial degradation thanks to its specific chemistry characterized by a wide C-to-N ratio, often above 100, coupled with a high aromatic level of organic carbon. As a consequence, estimated mean residence time of biochar in soils ranges from centuries to millennia (Lehmann, 2007), being several orders of magnitude higher than other soil amendments as crop residues (Bonanomi *et al.*, 2013), unprocessed agro-industry wastes (Nicolardot *et al.*, 2001), and compost (Gómez *et al.*, 2006). Recalcitrance to biological degradation makes especially attractive the possibility of converting waste biomass into biochar, and then use it as soil amendment to achieve an effective long-term carbon sequestration (Laird, 2008). In addition to the positive impact of biochar on carbon cycle on a global scale, the use of biochar

can positively affect crop performances. In a recent meta-analysis, Jeffery *et al.* (2011) showed that, on average, crop yields increase 10% after biochar application, with major benefits observed in soils having an acidic and neutral pH and in those with a coarse texture. Negative effects on crop performances have been reported only in a few cases (Wisnubroto *et al.*, 2010; Calderón *et al.*, 2015). The benefits of biochar on crop productivity have been related to four main mechanisms. First, since biochar is usually alkaline, a liming effect is often observed upon biochar amendments. The increase of soil pH, especially in acidic soils, can positively affect crop yields (Jeffery *et al.*, 2011). Second, because biochar has high water retention capacity, when incorporated into the soil, it improves water regime (Novak *et al.*, 2012). For instance, Barnes *et al.* (2014) found that biochar applications provide advantages in coarse grained, sandy soil as well in clay soil. In sandy soil, where water usually drains very quickly, biochar slowed the movement of moisture, thus reducing water loss, while in clay soil biochar promotes water drainage, reducing the risk of water-logging. The third mechanism is associated with the capability of biochar to adsorb and neutralize phytotoxic organic molecules including anthropogenic xenobiotics (Beesley *et al.*, 2011), as well as natural allelopathic compounds (Hille and den Ouden, 2005). The detoxifying capability is directly related to the dramatic increases of specific surface area that occur during pyrolysis (Chen *et al.*, 2008; Downie *et al.*, 2009). Thanks to this property, biochar has been proposed as a new tool for the reclamation of soils, sediments and also water contaminated by a range of organic pollutants including heavy metals (Ahmad *et al.*, 2014). Finally, the fourth mechanism explaining the benefits of biochar application on plant growth and health is related to its capability to stimulate beneficial microbes, in bulk soil as well as in the rhizosphere (Lehmann *et al.*, 2011; Thies *et al.*, 2015). Several studies reported an increase of microbial biomass (Liang *et al.*, 2010), mycorrhizal fungi (Warnock *et al.*, 2007), and plant-growth-promoting microbes (Graber *et al.*, 2010; Kolton *et al.*, 2011) as a result of biochar applications, with related changes in microbial community functionality.

In spite of the considerable research effort on aforementioned topics, the possibility of using biochar as a tool for an effective control of plant diseases has been mainly ignored. However, in recent years (Fig. 1A), evidence was reported that biochar application caused an effective suppression of diseases caused by both airborne (Harel *et al.*, 2012; Graber *et al.*, 2014) and soilborne plant pathogens (Elmer and Pignatiello, 2011). Here, we review available studies that investigate the effect of biochar on plant disease, the mechanisms behind disease suppression and identify research gaps and requirements for future studies. Specifically we focus on three main aspects: (i) the suppressive capacity of biochar types and the response of

different plant-pathogen systems, (ii) the analyses of the mechanisms behind biochar suppressiveness, and (iii) understanding the relationship between biochar sorption capability, soil quality and plant health.

Fig. 1. Number of papers published in the last 30 years on biochar (A) and compost (B) considering all studies (full dots) and only those related to suppression of plant diseases (open dots).



2.3 BIOCHAR SUPPRESSION OF PLANT DISEASES

In recent years, in order to develop a more eco-sustainable agriculture, research has been carried out on strategies for plant pathogen control characterized by high efficiency and limited environmental impact. In this context, the possibility of applying organic amendments including animal manure, green manure, organic wastes from agro-industry, and compost are of considerable interest, both among scientists (review in Hoitink and Boehm, 1999) and farmers. Organic amendments, besides improving soil structure and soil quality (Bonanomi *et*

al., 2014), can effectively suppress disease caused by both soilborne (Noble and Coventry, 2005) and airborne plant pathogens (Zhang *et al.*, 1996). Among organic materials, compost is the most studied (Fig. 1B) with many papers reporting effective disease suppression of pathogens such as *Fusarium* spp., *Phytophthora* spp., *Pythium* spp., *Rhizoctonia solani*, *Sclerotinia* spp., *Sclerotium* spp., and *Verticillium dahliae*. (review in Bonanomi *et al.*, 2007). However, despite the potential value of organic amendments there are several concerns about potential side-effects that limit practical applications of this technique. Some studies report that unprocessed organic materials as well as mature compost can enhance plant disease incidence and severity (Mazzola *et al.*, 2001; Tilston *et al.*, 2002; Yulianti *et al.*, 2006). Such negative effects were often associated with a release of phytotoxic compounds from decaying organic amendments (Cochrane, 1948; Patrick, 1971; Bonanomi *et al.*, 2006a), that may damage plant roots and predispose them to pathogen attacks (Patrick and Toussoun, 1965; Ye *et al.*, 2004; Bonanomi *et al.*, 2011a).

The beneficial effects of organic amendments, in the majority of the cases, largely outweigh their side-effects but, because of the lack of guidelines to predict the impact of the different types of amendments on different soils and pathosystems (Scheuerell *et al.*, 2005; Termorshuizen *et al.*, 2007; Bonanomi *et al.*, 2010), further research is needed for extensive and reliable applications. In this context, biochar appears as a new and promising tool to control a number of plant diseases. The studies that assessed the impact of different biochar types on plant diseases are summarized in Table 1. A total of 12 papers, with 18 experimental case studies assessing the effect of biochar on disease suppression, have been published. This number is very small compared to that available for other organic amendments (review in Bonanomi *et al.*, 2007). However, a progressive increase of published papers has been observed in the last few years (Fig. 1A, 1B). In general terms, the effect of biochar amendment was suppressive in 89% and non-significant in 11% of the case studies, respectively (Table 1). This frequency of effective disease suppression is much higher compared to that recorded for peat (4%), unprocessed crop residues (45%), but also for compost (56%) according to a meta-analysis based on data taken from 252 papers with 1964 experimental case studies (Bonanomi *et al.*, 2007).

Table 1. List of experimental studies that applied biochar as soil amendment for controlling plant diseases caused by airborne and soilborne plant pathogens. Pathogen, host plant, biochar feedstock type, mechanism and reference are reported for each study.

Pathogen	Host plant	Feedstock and pyrolysis condition	Proposed mechanisms of disease suppression	References
<i>Botrytis cinerea</i>	<i>Lycopersicon esculentum</i>	Citrus wood	Induced resistance in plants	Elad <i>et al.</i> , 2010
	<i>Capsicum annuum</i> cv. Maccabi	Citrus wood	Induced resistance in plants	Elad <i>et al.</i> , 2010
	<i>Fragaria x ananassa</i>	Citrus wood – crop wastes: 450 °C	Induced resistance in plants	Harel <i>et al.</i> , 2012
	<i>Lycopersicon esculentum</i>	Greenhouse wastes: 450 °C	Induced resistance in plants	Mehari <i>et al.</i> , 2015
<i>Colletotricum acutatum</i>	<i>Fragaria x ananassa</i>	Citrus wood - crop wastes: 450 °C	Induced resistance in plants	Harel <i>et al.</i> , 2012
<i>Fusarium oxysporum</i> f.sp. <i>asparagi</i>	<i>Asparagus</i> sp.	C. Quest Biochar (commercial product)	Sorption of phytotoxic compounds. Increased arbuscular mycorrhizal colonization	Elmer and Pignatello, 2011
	<i>Asparagus officinalis</i> cv. Mary Washington 500 W	Coconut charcoal - carbonized Chaff	Increased arbuscular mycorrhizal colonization	Matsubara <i>et al.</i> , 2002
<i>Fusarium oxysporum</i> f.sp. <i>radicis-lycopersici</i>	<i>Lycopersicon esculentum</i>	Pig bone: 550 °C	Biochar acts as a carrier for biological control agents	Postma <i>et al.</i> , 2013
<i>Fusarium proliferatum</i>	<i>Asparagus</i> sp.	C. Quest Biochar (commercial product)	Sorption of phytotoxic compounds. Increased arbuscular mycorrhizal colonization	Elmer and Pignatello, 2011
<i>Leveillula taurica</i>	<i>Capsicum annuum</i> cv. Maccabi	Citrus wood	Induced resistance in plants	Elad <i>et al.</i> , 2010
<i>Phytium aphanidermatum</i>	<i>Lycopersicon esculentum</i>	Pig bone: 550°C	Biochar acts as a carrier for biological control agents	Postma <i>et al.</i> , 2013
<i>Phytophthora cactorum</i>	<i>Acer rubrum</i>	Wood (<i>Pinus taeda</i> , <i>Pinus palustris</i> , <i>Pinus echinata</i> , <i>Pinus elliotti</i>): 550, 600°C	Induced resistance in plants	Zwart and Kim, 2012
<i>Phytophthora cinnamomi</i>	<i>Quercus rubra</i>	Wood (<i>Pinus taeda</i> , <i>Pinus palustris</i> , <i>Pinus echinata</i> , <i>Pinus Elliotti</i>): 550, 600°C	Induced resistance in plants	Zwart and Kim, 2012
<i>Plasmodiophora brassicae</i>	<i>Brassica rapa chinensis</i>	Miscanthus	No disease suppression observed	Knox <i>et al.</i> , 2015
<i>Podosphaera aphanis</i>	<i>Fragaria x ananassa</i>	Citrus wood – crop wastes: 450 °C	Induced resistance in plants	Harel <i>et al.</i> , 2012
<i>Ralstonia solanacearum</i>	<i>Lycopersicon esculentum</i>	Municipal waste biochar	No mechanisms identified	Nerome <i>et al.</i> , 2005
<i>Rhizoctonia solani</i>	<i>Cucumis sativus</i> cv. Muhasan	Eucalyptus wood chips- crop wastes: 350, 600 °C	No mechanisms identified. No direct biochar toxic effect on <i>R. solani</i>	Jaiswal <i>et al.</i> , 2014a
	<i>Phaseolus vulgaris</i>	Eucalyptus wood chips - crop wastes: 350, 600 °C	No mechanisms identified	Jaiswal <i>et al.</i> , 2014b

Biochar has been reported to be effective in suppressing diseases caused by both airborne and soil borne plant pathogens (Table 1). Concerning airborne pathogens, application of different biochar derived from citrus wood were able to control grey mould caused by *Botrytis cinerea* on *Lycopersicon esculentum*, *Capsicum annum* (Elad *et al.*, 2010) and *Fragaria x ananassa* (Harel *et al.*, 2012). Similar results were reported by Mehari *et al.* (2015) with biochar derived from greenhouse wastes that was able to control *B. cinerea* grey mould of *L. esculentum*. Elad *et al.* (2010) and Harel *et al.* (2012) reported that biochar produced from wood and greenhouse wastes under controlled conditions significantly reduced the incidence of powdery mildew caused by *Leveillula taurica* on *L. esculentum* and *Podosphaera aphanis* on *Fragaria x ananassa*, respectively.

In regard to soilborne pathogens, biochar suppressive capability has been reported for *Fusarium oxysporum* f. sp. *asparagi*, *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *Fusarium proliferatum*, *Pythium aphanidermatum*, *Phytophthora cactorum*, *Phytophthora cinnamomi*, and *Rhizoctonia solani* (Table 1). Two interrelated studies reported that biochar obtained from *Eucapiltus* wood and greenhouse waste was able to effectively control seedling damping-off caused by *R. solani* on *Cucumis sativus* (Jaiswal *et al.*, 2014a) and *Phaseolus vulgaris* (Jaiswal *et al.*, 2014b). These results deserve special interest because an effective control of *R. solani* with organic amendments, including composts, is notoriously difficult (Krause *et al.*, 2001; Scheuerell *et al.*, 2005; Termorshuizen *et al.*, 2007). For instance, Tuitert *et al.* (1998) reported that *R. solani* disease suppression was controlled by compost age: immature and very mature composts were suppressive, whereas at intermediate maturity the same compost was conducive to the disease. However, more than two positive cases are needed to demonstrate that biochar is an effective and reliable tool for controlling *R. solani*.

Overall, available evidence demonstrates that biochar can potentially control seven soilborne plant pathogens, a quite limited spectrum if compared with compost and crop residues that are potentially capable of controlling 79 soilborne pathogens (Bonanomi *et al.*, 2007). However, the restricted spectrum of plant diseases controlled by biochar likely reflects the limited research carried out so far on this topic. Future investigations on this emerging tool are needed to examine the capability of different biochar types to control diseases in other pathosystems. Most notably, only two studies reported an increase of disease incidence or severity, a disease conducive effect, upon soil amendment with biochar at the highest application rate tested (Jaiswal *et al.*, 2014a; Knox *et al.*, 2015). This general pattern contrasts with previous findings that crop residues and compost increase disease incidence when incorporated into soil in 28% ($n=586$) and 12% ($n=1016$) of the case studies, respectively

(Bonanomi *et al.*, 2007). Based on available evidence, biochar appears to be a very promising tool because it is often suppressive with only a few reported cases of conducive effects. However, publication biases for negative results could be especially relevant when the sample size is small, as it occurs for biochar ($n=18$) compared to crop residues ($n=586$) and compost ($n=1016$) (Bonanomi *et al.*, 2007). In this regard, available studies encompass only a small part of the wide spectrum of biochar types and potential pathosystems and further studies are required to evaluate the disease control capability of different biochar types.

2.4 BIOCHAR MECHANISMS BEHIND DISEASE SUPPRESSION

How can biochar control plant diseases? At least five different mechanisms have been proposed: (i) induction of systemic resistance in the host plants; (ii) enhanced abundance and/or activities of beneficial microbes; (iii) modification of soil quality in terms of nutrient availability and abiotic conditions; (iv) direct fungitoxic effect of biochar; (v) sorption of allelopathic, phytotoxic compounds.

Induced resistance in plants has been claimed as a putative mechanism for the control of disease suppression by biochar in 9 out of 18 experimental case studies analysed (Table 1). However, in 7 out of the 9 studies, only indirect evidence of induced resistance in plants was provided (e.g. Elad *et al.*, 2010; Zwart and Kim, 2012): biochar was applied in a place spatially separated from the infection sites, thus excluding the occurrence of other potential mechanisms in disease suppression. Harel *et al.* (2012) and Mehari *et al.* (2015) provided more direct evidence in support of the induced resistance hypothesis and suggested that both systemic acquired resistance (SAR) and induced systemic resistance (ISR) pathways were involved. Harel *et al.* (2012) reported that strawberry plants grown in substrates amended with biochar have a higher expression of genes encoding three pathogen related proteins (*FaPRI*, *Faolp2*, *Fra a3*), a gene that encodes a lipoxygenase (*Falox*), and a gene (*FaWRKY1*) encoding a trans-acting factor that belongs to the WRKY family. More recently, Mehari *et al.* (2015) demonstrated, by using the *S. lycopersicon* – *B. cinerea* pathosystem, that biochar-mediated ISR in tomato was dependent on jasmonic acid (JA). Specifically, by using tomato mutants modified in their salicylic acid, ethylene or JA metabolisms, the authors found that only JA deficient mutants prevent biochar induced resistance and limited H₂O₂ accumulation upon infection. However, the evidence reported by Harel *et al.* (2012) and Mehari *et al.* (2015) was not sufficient to clarify if induced plant resistance was due to a direct effect of

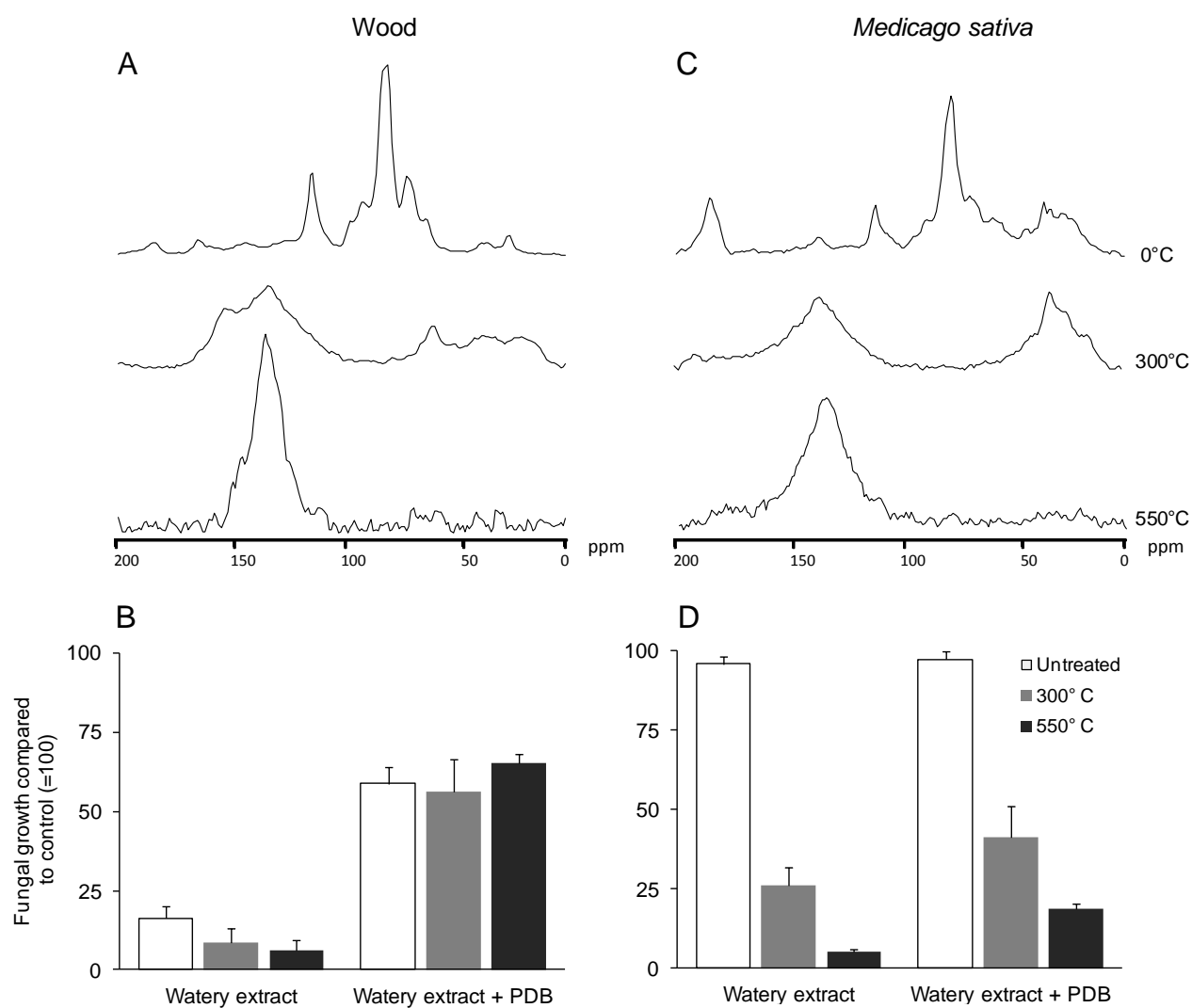
some chemical compounds present in biochar or to changes in rhizosphere microbioma composition and functions caused by the biochar amendment.

The second mechanism to explain disease suppression hypothesizes that biochar amendments can promote growth and/or activities of a range of beneficial microbes which, in turn, protect the plant from pathogen attacks. A growing body of evidence demonstrates that biochar increases microbial biomass (Liang *et al.*, 2010), root colonization by mycorrhizal fungi (Warnock *et al.*, 2007), and population of plant-growth-promoting microbes (Graber *et al.*, 2010; Kolton *et al.*, 2011). Such positive effects have been related to both physical and nutritional factors. First, thanks to its porous structure with high specific surface area, biochar offers a moist environment as well as safe sites for microbes from grazer such as mites, collembolan, protozoans and nematodes (Lehmann *et al.*, 2011). Empirical evidence indicates that both bacteria and mycorrhizal fungi are able to effectively exploit biochar porous structure (Downie *et al.*, 2009) to find refuge from predators (Warnock *et al.*, 2007). In regard to nutritional effect, biochar can provide organic carbon to sustain microbial saprophytic growth although this effect is expected to be very limited compared to other organic amendments such as crop residues and composts. In fact, as biomass is pyrolyzed its biochemical quality for microbe needs dramatically decreases because of the progressive disappearance of easily degradable carbon sources and enrichment of recalcitrant aromatic fractions (Krull *et al.*, 2009). As a consequence, after pyrolysis, biochar becomes an organic material suitable to sustain crop performances, but poorly capable of sustaining microbial growth. Recent results of our research group support this hypothesis. We made biochar at 300°C and 550°C from two feedstock: *Medicago sativa* hay, rich in organic nitrogen and carbohydrates, and wood chips that, in contrast, are rich in cellulose and lignin. The use of ¹³C-CPMAS NMR (Knicker, 2007) allowed a detailed monitoring of the chemical changes occurring in plant biomass following pyrolyzation. We found consistent and progressive losses of *O*-alkyl C and di-*O*-alkyl C, associated with carbohydrates, and a corresponding increase of aromatic carbons with increasing temperature of pyrolyzation (Fig. 2A, 2C). Such chemical changes affected saprophytic growth of some plant pathogenic fungi. We found that untreated biomass acts as a substrate to sustain fungal growth, especially *M. sativa* hay (Fig. 2B, 2D). On the contrary, a steady fungal growth reduction was recorded over plant biomass pyrolyzed at 300°C. Decreasing fungal growth was progressively confirmed at higher temperatures, with fungi almost completely inhibited by extracts of biochar made at 550°C, likely due to the progressive disappearance of easily degradable carbon sources and the concurrent enrichment of aromatic fractions (Fig. 2). All these results indicate that biochar

may be an interesting alternative to soil amendments with crop residues or composts because it selectively enhances the activity of beneficial microbes without stimulating pathogen populations and virulence. However, further investigations are required on this topic because, in contrast with the growing knowledge about the effects of biochar on soil microbioma (Lehmann *et al.*, 2011), only a few studies provide a direct link between biochar-induced changes in microbial community and effective disease suppression. A notable exception was the recent study by Postma *et al.* (2013) in which biochar made from animal bones was used as a carrier for an effective delivery of biocontrol agents. The authors showed, by scanning electron microscopy, that several bacteria (i.e. *Pseudomonas chlororaphis*, *Bacillus pumilus* and *Streptomyces pseudovenezuelae*) were able to extensively colonize the porous structure of biochar and when applied to the soil were most effective in controlling diseases caused by *Pythium aphanidermatum* and *F. oxysporum* f. sp. *lycopersici* on tomato.

The third possible mechanism is based on the hypothesis that modification of soil quality in terms of nutrient availability and abiotic conditions can affect the net result of plant-pathogen interactions. Biochar amendments usually ameliorate soil base content (i.e. Ca^{2+} , Mg^{2+} , K^{+}) (Gaskin *et al.*, 2010) and increase soil pH (Yuan and Xu, 2011). On the other hand, the impact on bioavailability of key plant nutrients such as nitrogen and phosphorus is more controversial (Chan and Xu, 2009). Because the pyrolysis process removes more nitrogen than organic carbon, the resulting biochar, depending on the temperature and initial biomass characteristics, usually has a C/N ratio higher than that of original feedstocks. When incorporated into soil, organic materials with a high C/N ratio stimulate microbial activity that, by reducing the availability of mineral nitrogen, impairs pathogen saprophytic capability and thus the infection process (Snyder *et al.*, 1959). At the same time, however, decomposition of organic materials with C/N ratio above the critical value of *ca.* 30 induces N starvation due to microbial competition that can impair also plant growth (Hodge, 2004). According to these considerations, biochar has a great potential to affect plant-pathogen interactions. However, to the best of our knowledge, no studies provide unambiguous evidence that demonstrate the link between biochar improvement of soil nutrient status or changes in soil abiotic conditions, e.g. liming effect, and an effective disease suppression. For instance, Knox *et al.* (2015) reported that biochar amendment increases soil pH but has no significant effect or, in some cases, even promotes clubroot disease of Chinese cabbage caused by *Plasmodiophora brassicae*.

Fig. 2 ^{13}C -CPMAS NMR spectra of untreated feedstock (wood powder and *Medicago sativa* hay) and the same feedstocks charred at 300°C and 550°C (A-C). Arrows indicate main chemical changes occurring during pyrolyzation of biomass. Degradation of di-*O*-alkyl and *O*-alkyl carbons, associated with sugars and carbohydrates including cellulose, and a contemporary neo-formation of aromatic carbon compounds were observed. (B-D) Responses of fungal pathogens exposed to watery extracts (50 g l⁻¹) of organic feedstock either untreated or charred at two different temperatures for five hours, with or without addition of potato dextrose broth (PDB). Data refer to fungal growth and are expressed as percentage of unexposed controls grown over PDB. Values are average of four fungal species (*Aspergillus niger*, *Fusarium oxysporum*, *Penicillium italicum*, and *Rhizoctonia solani*).



A further putative mechanism explaining disease suppression is the direct fungitoxic effect of biochar. During pyrolyzation of biomass, large chemical changes take place with degradation of *O*-alkyl carbons, associated with carbohydrate, and a contemporary neo-formation of aliphatic and aromatic C compounds (Fig. 2A, 2C). In addition, during pyrolysis

a range of organic compounds with potential fungitoxic are produced (Spokas *et al.*, 2011). However, studies that investigated the direct biochar fungitoxic effects reported only mild or no inhibition on fungal growth (Table 1). For instance, Jaiswal *et al.* (2014) found that different biochar types suppressed damping-off caused by *R. solani* on *Phaseolus vulgaris*. However, both *in vitro* and *in vivo* assays demonstrate that biochar has small or no significant direct inhibitory effect on *R. solani*. Similar results were found by our research group when testing *M. sativa* hay and wood biochars on hyphal growth of *Aspergillus niger*, *Fusarium oxysporum*, *Penicillium italicum*, and *Rhizoctonia solani* (Fig. 2B, 2D). All fungi thrive on *M. sativa* hay water extracts, while a lower growth was found on wood extract. However, growth of all tested microbes showed a steep decline on biochar (Fig. 2B, 2D). In our experiments, however, the addition of simple carbon sources (i.e. potato dextrose broth) to biochar extracts partially or completely restored microbial growth on *M. sativa* hay and wood biochar, respectively. These results support the hypothesis that biochar inhibition of microbial growth is mainly controlled by the availability of easily degradable carbon sources, with a minor role played by newly formed aromatic and phenolic compounds potentially fungitoxic. Our findings suggest that the effect of biochar could be the result of a balance between the availability of labile organic carbon sources and the presence of recalcitrant and/or fungitoxic compounds that provide little support or even inhibit microbial growth. In this context a notable methodological caveat is that the majority of the studies assessing biochar fungitoxic effects were done *in vitro*. Only the study by Jaiswal *et al.* (2014b), in which the impact of biochar amendment on *R. solani* population dynamics in soil was investigated, reported a small but statistically significant inhibition. In summary, available evidence suggests that direct biochar toxicity towards the pathogen can hardly explain the observed disease suppression. Similarly, Bonanomi *et al.* (2007) found that effective disease control by composts was not often (31% of 302 study cases) correlated with a significant reduction of the pathogen population. To better understand the impact of biochar on plant diseases, further research should investigate the impact of biochar on pathogen population dynamics in realistic ecological conditions.

2.5 BIOCHAR AS DETOXIFYING TOOL

The last mechanism proposed to explain biochar disease suppression is related to its capability of specifically adsorbing allelopathic, phytotoxic organic compounds. Biochar

sorption capability has several positive but also some negative implications that deserve specific attention for a successful use of this tool in different agricultural systems.

Carbonaceous sorbent materials such as activated carbon and biochar have a very large specific surface area, several hundred or even thousand fold greater than the source feedstock biomass (Chen *et al.*, 2008; Downie *et al.*, 2009). Thanks to this property, activated carbon, i.e. a form of carbon with increased specific surface area due to specific chemical treatments, is widely used in industrial processes as well as for medical applications and for decontamination of soils, sediments and especially wastewater. In this regard, biochar has been proposed as a substitute for activated carbon because it is cheaper and, in some cases, shows comparable adsorption properties (Oleszczuk *et al.*, 2012; Beesley *et al.*, 2011; Ahmad *et al.*, 2014).

In the context of plant pathology, biochar sorption capability can have direct as well as indirect beneficial functions. In agricultural soils, phytotoxic compounds can occur as a result of environmental pollution (Prasad, 2004), or can be released from decomposition of organic materials including crop residues (Patrick, 1971; Putnam and DeFrank, 1983), agro-industry organic wastes (Martin *et al.*, 2002), and immature compost (Tiquia, 2010). In this regards, biochar can directly protect plant roots from phytotoxic compounds actively released through root exudates of other plant species (Mahall and Callaway, 1992), or during decomposition of decaying plant residues (Bonanomi *et al.*, 2011b) and organic amendments (Table 2). A positive interaction between phytotoxic compounds released by decomposing organic materials and soilborne pathogens has been reported (Patrick and Toussoun, 1965; Blok and Bollen, 1993; Nigh, 1989; Ye *et al.*, 2004; Bonanomi *et al.*, 2006a; Bonanomi *et al.*, 2011a): plant roots under such abiotic stressful conditions can greatly increase their susceptibility to pathogens. Thus, by absorbing phytotoxic organic compounds, biochar can provide an indirect protection from the attack of soilborne pathogens. Compelling evidence in support of this hypothesis was provided by the study of Elmer and Pignatello (2011) by using the *Asparagus – Fusarium* crown and root rot pathosystem. Addition of biochar to soil promotes *Asparagus* root growth and determinates a reduction of root lesions caused by *F. proliferatum* and *F. oxysporum* f. sp. *asparagi*. Interestingly, in the presence of biochar, *Asparagus* plants showed a dramatic increase of colonization by arbuscular mycorrhizal fungi. This effect occurs because both plant roots and mycorrhizae were protected by biochar that adsorbed phytotoxic and fungitoxic phenolic compounds (e.g. cinnamic, coumaric and ferulic acids) released from decaying *Asparagus* crop residues. This study highlighted that biochar can have complex, multiple effects on the interaction between plant, pathogens and beneficial

microbes. Biochar, thanks to its sorption capability, can potentially modulate the interactions among microbes and between microbes and plants in the rhizosphere because it may interfere with their chemical-based signalling network. Recently, Masiello *et al.* (2013) found that wood biochar disrupts *Escherichia coli* cell-to-cell communication based on N-3-oxo-dodecanoyl-L-homoserine lactone, an acyl-homoserine lactone, by binding this signalling molecule that bacterial cells secrete to coordinate their activity. Of note, biochar made at 700°C has a capacity to interfere with *E. coli* 10-fold higher than that of biochar made from the same raw material at 300°C because of its much higher specific surface area. Further research is needed on biochar to fully evaluate its potential to modulate plant-microbe interactions, including nitrogen fixation and attack by soilborne bacterial pathogens.

Biochar sorption capability can also have relevant side-effects in specific agroecosystems. A number of studies reported that biochar may absorb many agrochemicals including herbicides, insecticides and fungicides (Table 2). On one hand, selective sorption of organic agrochemicals from soil can be beneficial because it reduces pesticide residues in vegetables (Table 2). Yang *et al.* (2010) reported that, in spite of a greater persistence of chlorpyrifos and fipronil residues in soils amended with biochar, the uptake by *Allium tuberosum* of two insecticides from amended soils was markedly lower than that from untreated control. On the other hand, in field conditions, since biochar addition to soil can greatly reduce agrochemicals efficacy (reviewed in Kookana, 2010), higher application rates are required to achieve the same control of pests. For instance, Graber *et al.* (2011a) reported that soil amendment at 2% with biochar reduces the capability of the herbicides S-metolachlor and sulfentrazone to control the grass *Setaria viridis*. Another paper of the same research group reported that corn-straw biochar reduced the efficacy of 1,3-dichloropropene against soil nematodes. This effect occurred as a result of a strong adsorption of the fumigant by biochar in both the vapor and aqueous phases of the soil (Graber *et al.*, 2011b). These results indicate that a preventive, careful evaluation of pros and cons of biochar application to soil should be done, also taking into account that, because of its long residence time, this material can cause durable changes in the physical and chemical properties of the soil. To avoid adverse impact of biochar on pest control through agrochemicals, special attention must be paid to the sorption strength of biochar, a property directly relate to its specific surface area, and the application rate in field conditions.

Table 2. List of experimental studies assessing the effects of biochar and activated carbon on agro-chemicals, plant derived allelochemicals and complex organic materials.

Compounds	Biochar feedstock and pyrolysis condition	Main effects	References
<i>Agro-chemicals</i>			
1,3-dichloropropene (fumigant)	Corn straw: 500°C	Effective sorption	Graber <i>et al.</i> , 2011b
4-chloro-2-methylphenoxy - acetic acid (herbicide)	Wheat straw	Increase sorption and reduced leachability	Tatarková <i>et al.</i> , 2013
Atrazine (herbicide)	Dairy manure: 100, 200, 300, 400, 500 °C	Effective sorption from water	Cao and Harris, 2010
	Dairy manure: 450 °C	Effective sorption from soil	Cao <i>et al.</i> , 2011
Atrazine and Acetochlor (herbicides)	C. Quest Biochar (commercial product): 500°C	Effective sorption	Spokas <i>et al.</i> , 2009
Atrazine and Simazine (herbicides)	Green waste: 450 °C	Effective sorption and partition from water	Zheng <i>et al.</i> , 2010
Chlorpyrifos and Carbofuran (insecticides)	Woodchips: 450, 850 °C	Immobilization from soil that increases with biochar microporosity	Yu <i>et al.</i> , 2009
Chlorpyrifos and Fipronil (insecticides)	Cotton straw: 450, 850 °C	Immobilization from soil that increases with biochar microporosity	Yang <i>et al.</i> , 2010
Deisopropylatrazine (herbicide)	Broiler litter: 350, 700 °C	Effective sorption	Uchimiya <i>et al.</i> , 2010
Diuron (herbicide)	Woodchips: 450, 850 °C	Effective sorption	Yu <i>et al.</i> , 2006
Norflurazon and Fluridone (herbicides)	Wood and grass: 200, 300, 400, 500, 600 °C	Effective sorption from water	Sun <i>et al.</i> , 2011
Pentachlorophenol (pesticide)	Rice straw - temperature not reported	Effective sorption from soil that increases with surface area and microporosity	Lou <i>et al.</i> , 2011
	Bamboo: 600 °C	Reduced leachability and bioavailability from soil	Xu <i>et al.</i> , 2012
Pyrimethanil (fungicide)	Red gum woodchips: 450, 850 °C	Immobilization from soil that increases with surface area and microporosity	Yu <i>et al.</i> , 2010
Simazine (herbicide)	Hardwood: 450, 600 °C	Reduced leachability and bioavailability from soil	Jones <i>et al.</i> , 2011
S-metolachlor and Sulfentrazone (herbicides)	Wood charcoal, Eucalyptus wood: 800 °C	Reduced efficacy	Graber <i>et al.</i> , 2011a
<i>Allelopathic compounds and complex organic materials</i>			
Compost	Hardwood-derived biochar	Effective alleviation of phytotoxicity	Beesley <i>et al.</i> , 2010
Contaminated sediment	Corn stover - straw	Effective alleviation of phytotoxicity	Joško <i>et al.</i> , 2013
Crop residues	Several biochar	Effective alleviation of	Rogovska <i>et al.</i> ,

		phytotoxicity	2012
Plant litter	Activated carbon	Effective alleviation of phytotoxicity	Marler and Dongol, 2013
	Activated carbon	Effective alleviation of phytotoxicity	Mazzoleni <i>et al.</i> , 2015
	<i>Empetrum hermaphroditum</i> twigs: 450 °C	Effective alleviation of phytotoxicity	Wardle <i>et al.</i> , 1998
	Natural charcoal	Effective alleviation of phytotoxicity	Zackrisson <i>et al.</i> , 1996
Plant litter extracts	Activated carbon	Effective alleviation of phytotoxicity	Bonanomi <i>et al.</i> , 2011
	Activated carbon and natural charcoal	Effective alleviation of phytotoxicity	Hille and den Ouden, 2005
	Activated carbon	Effective alleviation of phytotoxicity	Hovstad and Ohlson, 2008
	Activated carbon	Effective alleviation of phytotoxicity	Jäderlund <i>et al.</i> , 1996
Root exudates	Activated carbon	Effective alleviation of allelopathic factors	Mahall and Callaway, 1992
Sewage sludge	Activated carbon, maize stover biochar: 600°C, straw biochar: 700°C	Effective alleviation of phytotoxicity	Oleszczuk <i>et al.</i> , 2012

2.6 CONCLUSIONS AND FURTHER PERSPECTIVES

In the last 10 years an impressive number of studies have been carried out on the subject of biochar, but research concerning the use of biochar for the control of plant diseases is still limited. Potential strategies for the adoption of biochar as a soil amendment are actually constrained by the scarcity of field-scale experiments, with the majority of studies carried out in laboratory or greenhouse conditions over a short period of time, often using very high application rates. Nevertheless, the evidence so far available indicates that biochar is a promising tool also for its low cost and the complementary positive effects on the environment, including the potential reduction of fertilizers and soil carbon storage. There is no doubt that biochar has beneficial effects on carbon storage, crop yields and in some cases on the protection of plants from pathogen attack and abiotic stress factors. However, to make significant scientific progress towards a better understanding of the biological nature of disease suppression achieved by biochar application in different plant-pathogen systems, research efforts should be focused on the following topics:

1. biochar has been tested only in a few pathosystems (Table 1). More research is needed to extend basic knowledge about disease suppression capability of biochar on new pathosystems;

2. biochar diversity can be exceptionally high in relation to initial feedstock biomass characteristics and pyrolysis conditions. Further experiments can extend our understanding of how different types of biochar affect plant-pathogen interactions;
3. available evidence suggests that biochar can selectively enhance the activity of beneficial microbes, without stimulating pathogen populations and virulence. Studies that test this hypothesis are needed;
4. the majority of available studies were done in laboratory or greenhouse conditions, with few cases addressing the potential application at field scale in realistic agricultural systems;
5. to improve our knowledge of the relationships between chemical and physical characteristics of biochar and pathogenic microbes in order to identify biochar quality parameters that consistently predict its suppressive capability.

Rapid answers to these questions are needed to make biochar a safe, effective and affordable tool for the control of plant pathogens.

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Chapter 3

Heat treated litter causes opposite effects on the growth of plants and fungi

3. HEAT TREATED LITTER CAUSES OPPOSITE EFFECTS ON THE GROWTH OF PLANTS AND FUNGI

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3.1 ABSTRACT

Fire, besides affecting plant litter fate in natural ecosystems, is a widely used tool to manage crop residues in agro-ecosystems. In both cases, burning of plant residues produces highly heterogeneous materials, ranging from little affected plant tissues, to charred substrates, up to mineral ashes, whose chemical nature and biological effects are not yet fully clarified. The aim of our study is to assess the effects of litter burned at different temperatures (100, 200, 300, 400 and 500 °C) on saprotrophic fungi, plant pathogenic microbes, and higher plant. To this purpose we combined a characterization of 48 organic materials by ^{13}C CPMAS NMR spectroscopy with a multi-species bioassay on seven target organisms (one plant and six microbes). We showed that, as heating temperature increases, litter quality significantly changes, with a progressive loss of O-alkyl C, di-O-alkyl C, and methoxyl and N-alkyl C, , coupled with an enrichment in aromatic carbons, irrespective of plant litter type. The bioassay showed that unheated litter has a major inhibitory effect on the test plant, while it acts as a suitable substrate sustaining microbial growth. On the contrary, as litter is heated its biochemical quality decreases with increasing temperature because of a progressive disappearance of easily degradable C sources and enrichment of recalcitrant aromatic fractions. As a consequence, heated litter becomes an organic material suitable to sustain plant growth, but is largely inhibitory for microbial performances. This work demonstrates that defining litter quality by ^{13}C -CPMAS NMR improves our understanding of the substrate preferences of both plant and microbes, for different litter types as well as for charred organic materials.

Keywords: Crop residues · Vegetation fire · Ash · Biochar · Soilborne pathogens · *Aspergillus* · *Pythium* · *Rhizoctonia* · *Trichoderma* · ^{13}C -CPMAS

3.2 INTRODUCTION

Plant litter represents an important fraction of primary ecosystem productivity reaching the soil (De Deyn et al., 2008). Litter is a key component of soil organic carbon in terrestrial ecosystems because its physical, chemical and biological breakdown releases nutrients that sustain plant growth, contribute to soil organic matter formation, and affect carbon balance (Aerts, 1997). Beside the well established role of temperature (Davidson and Janssens, 2006) and moisture (Liu et al., 2006), litter chemical quality (Fierer et al., 2005) is a crucial factor for controlling the fate of organic carbon.

In terrestrial ecosystems plant litter undergoes different pathways to complete mineralization, such as biological decomposition (Berg and McClaugherty, 2008), abiotic transformation through UV radiation (Austin and Vivanco, 2006), and physico-chemical transformations due to fire passage (Bond and Keeley, 2005). Fire affects carbon balance in several ecosystems including the Mediterranean biome (Pausas and Vallejo, 1999). Vegetation burning has relevant ecological impacts because affects CO₂ release in the atmosphere and produces a substantial input of newly formed carbon forms which are delivered into soil system (González-Pérez et al., 2004). Fire has been used for millennia by humans as tool for management of agro-pastoral systems (Kumar and Goh, 1999; Lal, 2005). For instance, it is largely used for a rapid and effective removal of slow-decomposing crop residues, such as orchard pruning, and for the elimination of infected tissues to limit spread of plant diseases (Hardison, 1976; Bonanomi et al., 2007).

In this context, it is relevant that burning of both natural vegetation and crop residues produce highly heterogeneous materials, ranging from little affected plant tissues, to a variety of charred substrates, until mineral ash (Johnson, 1996). The resulting composition of organic residues after a fire depends on both the biochemical composition of plant tissues (Knicker et al., 2008) and fire intensity that, in turn, is controlled by litter type, pre-fire biomass moisture, fuel spatial arrangement and local microclimatic conditions (Whelan, 1995; Thonicke et al., 2001). Several studies by using throughput analytical techniques such as pyrolysis-gas chromatography/mass spectrometry (Nocentini et al.; 2010) and ¹³C-CPMAS nuclear magnetic resonance (NMR) spectroscopy (Kögel-Knabner, 2002; Almendros et al., 2003) revealed that substantial changes occur above 270°C: plant litter rapidly reduces its nutrients (Gray and Dighton, 2006), decreases the content of carbohydrates and proteins and becomes progressively enriched in aromatic carbon compounds (review in Knicker, 2007). Moreover, several studies reported that temperatures ranging from 250°C to 350°C produce a relative

increase of aliphatic compounds (Robichaud and Hungerford, 2000; Almendros et al., 2003) which enhance soil water repellency (DeBano, 2000; Bodí et al., 2011). However, at higher temperatures aliphatic compounds rapidly disappear, with the same fate common to all unprotected organic carbons that are lost under oxic conditions at temperatures over 460°C (Knicker, 2007).

The biochemical changes of burned plant tissues can, potentially, affect plant growth and microbial activity. For instance, the inhibitory effect on plant root growth by undecomposed litter is a well established effect (Bonanomi et al., 2011a; Lopez-Iglesias et al., 2014; Meiners, 2014; Mazzoleni et al., 2015), while charred plant residues often promote plant growth (Jeffery et al., 2011), as a result of their sorption capability towards allelopathic compounds such as phenols (Zackrisson et al., 1996). On the other hand, a number of field studies highlighted the impact of fire and vegetation burning on soil functionality (Wardle et al., 2008), microbial succession and turnover (Vázquez et al., 1993; Visser, 1995; Persiani et al., 2011). Only few studies focused on the direct effects of charred litter on saprotrophic, mycorrhizal, and pathogenic fungi (review in Lehmann et al., 2011). All these pieces of evidence suggest a key role of litter and char biochemistry as explanatory factors of the effects of fire on both microbes and higher plants. Previous studies separately assessed litter (e.g. Bonanomi et al., 2011a; Meiners, 2014) or char effects on higher plants (Jeffery et al., 2011) and bacteria and fungi (Lehmann et al., 2011). Moreover, a number of studies provided a deep understanding of the biochemistry of charred material produced during plant biomass burning (Almendros et al., 2003; Knicker et al., 2005), but did not address their potential biological effects. In this study, to clarify the effect of litter burned at different temperatures on saprotrophic fungi, plant pathogenic microbes, and higher plant, we combined plant litter and charred materials characterization by ^{13}C -cross-polarization magic angle spinning (CPMAS) nuclear magnetic resonance (NMR) spectroscopy (Kögel-Knabner, 2002), with a multi-species bioassay approach. In detail, the effect of 48 types of organic materials, derived from litter of eight plant species heated at six temperature levels, was assessed on seven target species (one plant and six microbes). Specific aims of the study were:

- (1) to reveal the chemical changes occurring on different litter types when heated at increasing temperatures by using ^{13}C -CPMAS NMR spectroscopy;
- (2) to assess the effect of heated litter on growth of microbes and higher plants;
- (3) to explore the relationships between litter biochemical quality defined by ^{13}C NMR spectroscopy and growth of target species.

3.3 MATERIALS AND METHODS

3.3.1 *Plant material collection and heating treatment*

Eight different plant litter types were selected to represent a wide range of organic matter quality (see the section “Organic materials ^{13}C -CPMAS NMR characterization”). The eight litter types have very different values of C/N ratio, percent of nitrogen (N) and lignin content (Supplementary Table S1). Freshly abscised leaves were collected by placing nets under plants of natural communities (n° plants >20 , individuals randomly selected for each species). Litter materials were dried at room temperature in a ventilated chamber until constant weight was reached and then stored at room temperature.

Dry samples of 50 g of each species were subject to dry heating at five different temperatures (100, 200, 300, 400 and 500°C) for thirty minutes in a muffle furnace to obtain 48 different organic substrates (8 litter species \times 5 temperature levels plus eight untreated materials).

3.3.2 *Plant and fungal bioassays*

The bioassays were aimed at assessing the effects of untreated and heated litters on the growth of the plant *Lepidium sativum* (Bonanomi et al., 2011a) as well as on selected microbial species. A sub-set of the 30 organic materials was used in the bioassay (five litter types i.e. *Eruca sativa*, *Hedera helix*, *Medicago sativa*, *Pinus halepensis*, and *Quercus ilex*, either untreated or heated at the different temperatures). To obtain litter water extracts for both plant and fungal bioassays, dried organic material was mixed with distilled water in a beaker at 5% of dry weight (50 g l^{-1}) and shaken for 5 h. The aqueous suspensions were then centrifuged (2395 g for 10 min), sterilized (microfiltration with 0.22- μm pore filter) and stored at -20°C until bioassay.

The plant bioassay, hereafter called the “seed germination” experiment, was carried out on *L. sativum* as a target species because of its recognized sensitivity to phytotoxic compounds (Bonanomi et al., 2011a). The use of one test plant has the advantage of standardizing the results of different organic matter types. Briefly, 20 seeds were placed in 9 cm Petri dishes over sterile filter papers with 4 ml of test solution. The aqueous suspensions were diluted by distilled water to three concentrations (50, 17 and 5 g l^{-1}) and stored at -20°C until bioassay. Every solution plus the control with distilled water were replicated 3 times for

a total of 5,460 seeds for the whole experiment. Petri dishes were arranged in a growing room in a completely randomized design and seedling root length was measured 36 hours after germination.

The microbial bioassay was performed to assess the effects of organic materials on the saprotrophic growth of five fungi (i.e. *Aspergillus niger*, *Ganoderma lucidum*, *Penicillium italicum*, *Rhizoctonia solani*, and *Trichoderma harzianum*) and an oomycete (*Pythium ultimum*) with different functional features (Supplementary Table S2). This approach specifically allows to evaluate the relationships between litter biochemistry on microbial saprophytic growth. All strains were available at the mycology laboratory of Department of Agriculture and maintained on potato dextrose agar (PDA, Oxoid) medium. For three fungi (*A. niger*, *P. italicum*, and *T. harzianum*) we used a spore germination bioassay. Briefly, the inoculum was obtained by adding 10 ml of sterile water to ten day old cultures, grown in Petri dishes containing PDA, and by scraping the culture surface to remove conidia. The suspension was filtered, centrifuged, washed twice with sterile water and adjusted to a concentration of 10^5 conidia ml^{-1} by hemocytometer. Spore suspension was prepared in 10 μl of sterile water. Organic materials extracts were applied in 96-well plates and incubated at 24°C. Fungal growth was measured spectrophotometrically ($\lambda = 590 \text{ nm}$) after 20 hours of incubation using a Thermomax microtitre plate reader (Molecular Devices, Wokingham, UK). For *G. lucidum*, *P. ultimum*, and *R. solani* which do not produce conidia, hyphal growth bioassay was performed. Since the aim of this experiment was to test the ability of the microbes to use organic material as the single source of nutrient, microbial growth started from a water agar (WA, Oxoid) nutrient-poor medium. After seven days of culture on WA, a 4 mm diameter plug was collected from the edge of the growing colony and placed in the centre of the Petri dish. For such microbes substrates were prepared by mixing WA and sterile litter extracts at 1:1 ratio. Ten millilitres of each dilution were placed in a 9 cm Petri dish. PDA was used as the control substrate. Ten replicates were used for each treatment. After 72 h, hyphal density and radial growth of each colony were measured on five randomly chosen points. Hyphal density was measured by counting the number of hyphae crossing a 1 mm line at 250 \times magnification under binocular microscope. A growth index was calculated as the product of the area of fungal colony, calculated from the measured colony radius, and the hyphal density, following Tuitert et al. (1998).

3.3.3 *¹³C-CPMAS NMR characterization of organic materials*

The 48 organic samples (eight litter types either untreated or heated at five temperatures) were characterized by ¹³C-CPMAS NMR obtained in solid state under the same conditions in order to perform a quantitative comparison among spectra. The spectrometer used was a Bruker AV-300 equipped with a 4 mm wide-bore MAS probe. NMR spectra were obtained with MAS of 13000 Hz of rotor spin, 1s of recycle time, 1 ms of contact time, 20 ms of acquisition time, 2000 scans. Samples were packed in 4 mm zirconium rotors with Kel-F caps. The pulse sequence was applied with a 1H ramp to account for non-homogeneity of the Hartmann-Hahn condition at high spin rotor rates. Each ¹³C-CPMAS NMR spectrum was automatically integrated to calculate the area of the peaks which appeared in the chosen region. Spectral regions have been selected and corresponding C-types identified by previous reference studies (Kögel-Knabner, 2002; Bonanomi et al., 2011a): 0-45 ppm = alkyl C; 46-60 ppm = methoxyl and N-alkyl C; 61-90 ppm = O-alkyl C; 91-110 ppm = di-O-alkyl C; 111-140 ppm = H- and C- substituted aromatic C; 141-160 ppm O-substituted aromatic C (phenolic and O-aryl C); 161-190 ppm carboxyl C.

3.3.4 *Data analysis*

For statistical analysis of the bioassay results, the species response data were expressed as percentage of the respective control and submitted to Generalized Linear Models (GLMs) analysis, using the software STATISTICA 7 (StatSoft Inc., Tulsa, OK, USA). In detail, GLMs were used for plant bioassay considering main and interactive effects of litter type (5 levels), extract concentration (3 levels) and heating temperature (included as a continuous covariate). GLMs were run in the same way but separately for each microbial species (5 fungi and an oomycete) considering main and interactive effects of litter type (5 levels), extract concentration (2 levels), and litter heating temperature (continuous covariate). A further GLM was run with data pooled for all microbes, including main and interactive effects of microbial species and litter treatments. Pairwise differences were tested using Tukey's HSD post-hoc test. One-way ANOVA was used to assess the effect of heating temperature (6 levels) on litter chemical quality assessed by ¹³C CPMAS NMR. The tested null hypothesis was that litter heated at different temperatures, showing equal signal intensity within different spectral region, have an equal relative content of the corresponding C types.

To address the relationship between species performance in the bioassays and plant litter biochemistry, linear correlation was extensively calculated between species growth

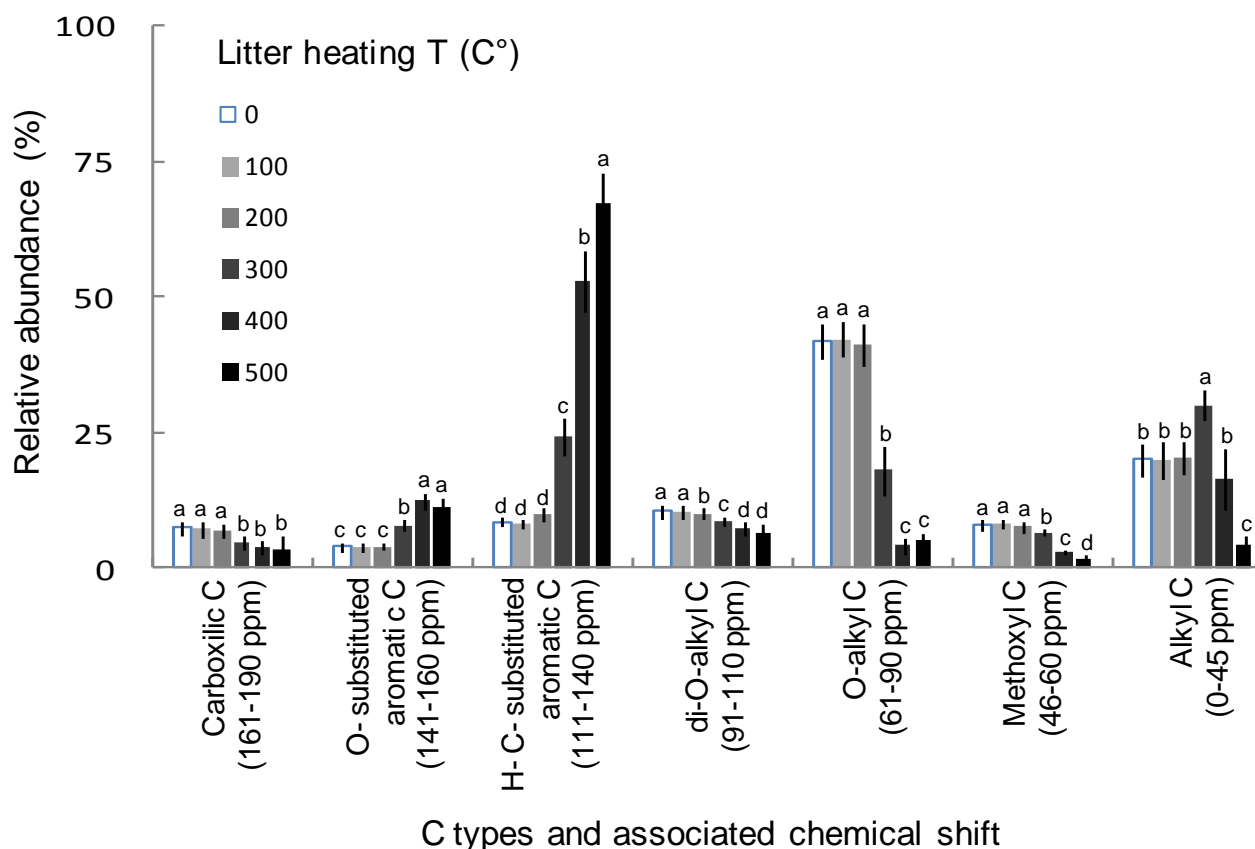
performance over the 30 litter extracts (5 litter types at $50 \text{ g l}^{-1} \times 6$ heating temperatures) and ^{13}C NMR data recorded for the same litter materials. Species performance was tested for correlation with ^{13}C -CPMAS NMR spectral regions ($N = 7$) selected from reference literature (Almendros et al., 2000; Kögel-Knabner, 2002; Bonanomi et al., 2011a; Pane et al., 2011) and with each resonance signal ($N = 200$), providing a finer-resolution profile of the C-types variation in the tested litter materials associated with the effect on the growth performance of plant and microbes. In order to control for multiple comparisons, correlation was tested for statistical significance at $\alpha = 0.05/N$, with N being the number of performed correlation tests, by applying the Bonferroni's correction.

3.4 RESULTS

3.4.1. Litter chemical changes associated with thermal treatments

The ^{13}C -CPMAS NMR spectra revealed significant and consistent changes of litter carbon types for all studied species, with major chemical changes progressively occurring at increasing heating temperatures (Figs. 1 and 2, Supplementary Fig. S1). Four spectral intervals, corresponding to O-alkyl-C, di-O-alkyl-C, methoxyl and N-alkyl C as well as the carboxylic region showed a progressive decrease when litter was treated at the temperature of 300°C (Fig. 1). The relative decrease was especially marked for the O-alkyl-C (61-90 ppm), mainly associated with sugars and polysaccharides, that largely decreased at 300°C and almost disappeared when litter was treated at 400°C and 500°C (Figs. 1 and 2). The aliphatic alkyl-C (0-45 ppm, characteristic of lipid waxes, cutins and microbial products) remained unaffected when litter was treated at 100°C and 200°C . However, a significant increase for this carbon signals was recorded for litter treated at 300°C , followed by a substantial decrease at 400°C and 500°C (Fig. 1). Aromatic regions (111-140 ppm and 141-160 ppm), in contrast with other spectral regions, increased when litter was heated at 300°C , and even more at 400°C and 500°C (Figs. 1 and 2). The O-substituted aromatic C (141-160 ppm) showed a slight, but significant increase when treated at temperatures above 300°C (Fig. 1). Differently, the H- and C- substituted aromatic C regions (111-140 ppm) sharply increased at 300°C in litter of *Medicago* and *Eruca*, with a further significant increase at 400°C and 500°C for all litter species (Figs. 1 and 2).

Fig. 1. Variation of relative abundance of seven main classes of organic C assessed by ^{13}C -CPMAS NMR spectroscopy in eight plant litter types treated at six different temperatures for 30 minutes. Data refer to mean values ($n=8$) \pm SD. Different letters within each class of organic C indicate temperature-dependent significant differences (Tukey's HSD post-hoc test from one-way ANOVA at $P < 0.05$).



3.4.2. Effects of thermally treated litter on plant and microbes

In the “seed germination” bioassay, litter type, heating temperature, and extracts concentration, as well as their interactions, had a significant effect on the growth of *L. sativum* (Supplementary Fig. S2 and Table S3). At the highest extract concentration, unheated plant litter showed a remarkable inhibitory effect on root growth, mostly independent of the litter type (Fig. 3A). Such an effect remained almost unaltered when litter was treated at 100 °C, but the inhibition progressively decreased at higher heating temperatures, with different magnitudes for different litter types (Fig. 3A). At 200 °C a slight release of the inhibitory effects was observed for most litters, with the exception of *H. helix* (Fig. 3A). The inhibitory effect of plant litter largely decreased at 300 °C, although in some cases stimulatory effects were found, for extracts of litter heated at 400 °C and 500 °C (Fig. 3A). After the treatment at 500 °C, *E. sativa* litter showed a clear stimulatory effect, *Q. ilex* litter produced an effect not significantly different from that of the control, while litter from

H. helix and *P. halepensis* displayed a consistent reduction of inhibition, and *M. sativa* a limited but significant reduction (Fig. 3A).

In spore germination and hyphal growth bioassays, response pattern of microbes to extracts of heated litter was completely different from that observed for plant growth. *A. niger*, *P. italicum*, *T. harzianum* and, to a lesser extent, *G. lucidum* all grew well over non-heated plant litters, mostly independent of litter types (Fig. 3, Supplementary Table S3). After a growth reduction over litters heated at 100 °C and 200 °C, variable according to litter types, a significant, steady growth reduction was recorded for these four fungi in the presence of all litter treated at 300°C. Such a trend of decreasing growth was progressively confirmed at higher temperatures, with the four fungi completely inhibited over extracts of all litter types treated at 400°C and 500°C (Fig. 3).

On the other hand, *R. solani* and *P. ultimum* presented temperature-dependent responses largely affected by different litter types (Fig. 3B, F). In both cases the response pattern was consistent with that of the other fungi over litter of *H. helix*, *E. sativa*, and *M. sativa*, but with remarkable differences in magnitude. The two latter materials, treated at 100 °C, showed a strong stimulatory effect on the growth of *R. solani* (Fig. 3B). Conversely, the oomycete *P. ultimum* showed variable responses at intermediate temperatures. However, both microbes were highly inhibited over litter heated at the temperature of 300°C or more. Interestingly, both *R. solani* and *P. ultimum* were mostly inhibited over extracts of *P. halepensis* and *Q. ilex* litter, regardless of the thermal treatment (Fig. 3B, F).

3.4.3. Organic material quality and target species sensitivity

The extensive analysis of correlation between species responses to litter extracts and all ¹³C-CPMAS NMR spectral signals of the same litter materials, revealed several resonance regions significantly correlated with growth of the target organisms (Fig. 4). The correlation profiles for plant and microbes were strikingly contrasting (Fig. 4). In the case of *Lepidium*, root growth was negatively and positively correlated with restricted resonance regions at 65-75 ppm, and at 125-145 ppm, respectively (Fig. 4). Considering the spectral regions derived from the literature, root growth was negatively correlated with O-alkyl C and methoxyl C regions, whereas significant positive correlations were found with aromatic C regions (Table 1).

In the case of the six microbes, an exceptionally homogeneous response to litter biochemical quality, as defined by ¹³C CPMAS NMR, was found (Fig. 4). The performance

of all species was positively associated with restricted resonance regions at 70-75 ppm, 105-110 ppm and 175-185 ppm and negatively with a wide section (120-155 ppm) of the aromatic C regions (Fig. 4). This general pattern showed only slight interspecific differences, as in the cases of *Pythium* and *Ganoderma*, that displayed a lower correlation magnitude compared with other fungi in the O-alkyl C and aromatic C regions (Fig. 4). Considering spectral regions derived from the literature, hyphal growth or spore germination of all microbial species were negatively associated with the two aromatic C regions, whereas significant positive correlations were found with methoxyl C and O-alkyl C regions (Table 1). Finally, the growth of *Aspergillus*, *Pythium*, *Rhizoctonia*, and *Trichoderma* was positively associated with the carboxylic C resonance region (Table 1).

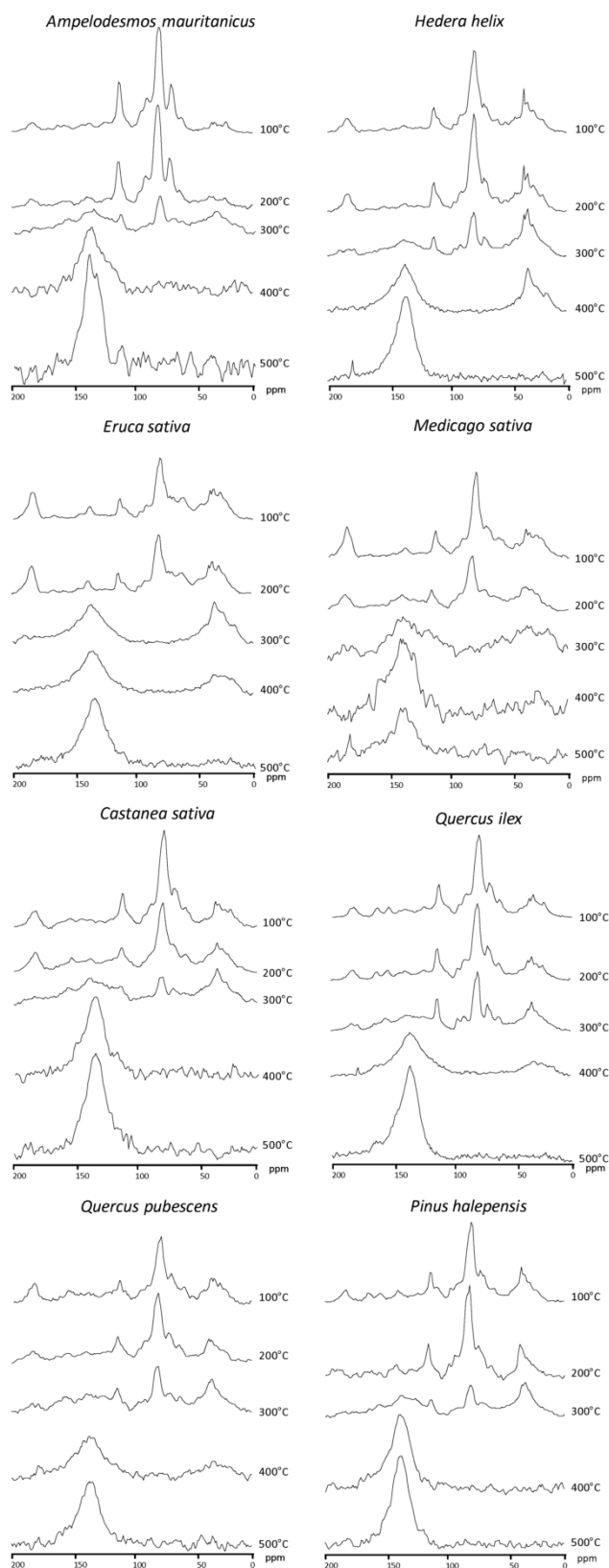
3.5 DISCUSSION

In this work we showed that the use of ^{13}C -CPMAS NMR allowed a detailed monitoring of the chemical changes occurring in litter during the heating treatment. We found consistent and progressive losses of O-alkyl C, di-O-alkyl C as well as methoxyl and N-alkyl C, for all types of plant litter, with corresponding increases of aromatic carbons. In addition, we demonstrated that undecomposed litter has a major inhibitory effect on the test plant, while acting as a substrate to sustain microbial growth. On the contrary, as litter is heated at high temperatures its biochemical quality decreases because of a progressive disappearance of easily degradable C sources and enrichment of aromatic fractions. As a consequence, after heating, litter becomes an organic material suitable to sustain plant growth, but largely inhibitory for fungal and oomycete performances. Finally, we demonstrated that defining litter quality by ^{13}C -CPMAS NMR provides a significant tool for understanding the substrate preferences of the tested species.

3.5.1. Litter chemical changes associated with thermal treatments

Plant litter heated at 100°C and 200°C showed barely detectable changes by ^{13}C CPMAS NMR analysis. However, as temperature reaches 300 °C the biochemical composition of all litter types undergoes dramatic changes. These were surprisingly similar for the eight litter types, regardless of the wide range of their chemical quality (i.e. lignin content and C/N ratio varied from 2.31% and 9.12% for *Eruca* and *Medicago*, respectively, to 21.72% and 41% for *Q. pubescens* and *A. mauritanicus*, respectively).

Fig. 2. ^{13}C -CPMAS NMR spectra of *Ampelodesmos mauritanicus*, *Castanea sativa*, *Eruca sativa*, *Hedera helix*, *Medicago sativa*, *Pinus halepensis*, *Quercus ilex*, and *Quercus pubescens* plant litter heated at five different temperature levels for 30 minutes. Spectra obtained for non-heated plant litters, as not significantly different from those of 100°C-heated materials (Fig. 1), are not shown.



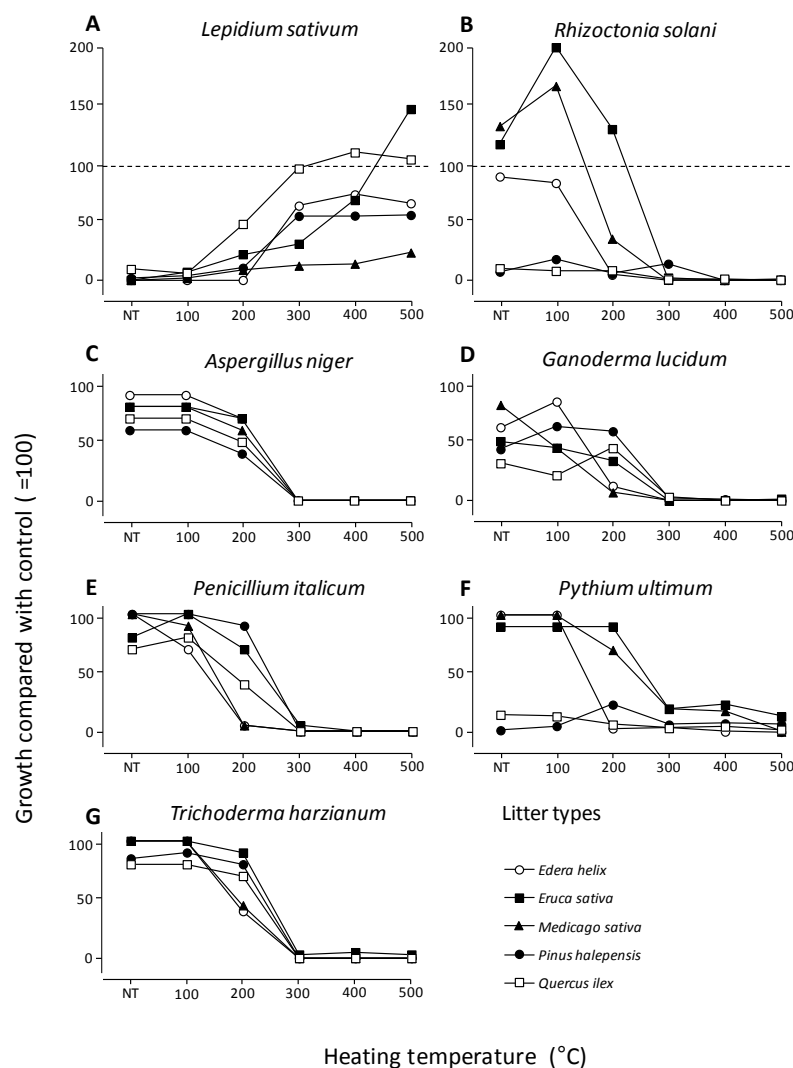
At 300°C, degradation of O-alkyl C, carboxyl C, and methoxyl C and a contemporary neo-formation of aliphatic and aromatic C compounds were observed. Firstly, the O-alkyl C fraction, mainly related to carbohydrates, vanished in *Eruca* and *Medicago*, sharply decreased in *Ampelodesmos*, *Castanea*, *Hedera* and *Pinus*, and showed a limited, yet still significant decrease in the two *Quercus* species (i.e. *Q. ilex* and *Q. pubescens*). A rapid depletion of the O-alkyl C fraction has been reported for both peat (Almendros et al., 2003) and grass residues (Knicker et al., 2005) treated at 350°C for few minutes. Our results, however, highlight that at lower temperatures the sensitivity of the O-alkyl C fraction to thermal degradation is inversely related to litter lignin content, i.e. the higher the lignin content, the lower is carbohydrates degradation. This result could be partially explained by the large proportion of plant sugars and cellulose entrapped in lignin (Adair et al., 2008), so that temperature degradation cannot proceed independently of that of lignin, which requires higher temperatures (González-Pérez et al., 2004). All litter treated at 300°C showed a significant increase of the alkyl fraction, mainly related to aliphatic compounds (Fig. 1), which was more evident for the grass *Ampelodesmos*, the nitrogen fixing *Medicago* and the coniferous tree *Pinus* (Supplement Fig. S1). In general, these results confirm the formation of a water repellent layer due to aliphatic compounds in soils after fire events (DeBano, 2000). Neo-formation of aliphatic compounds for thermally treated soils (Almendros et al., 1998; Knicker et al., 2005) and peat (Almendros et al., 2003) has been reported and related to condensation of water repellent, aromatic polymers. However, also the more stable, paraffin like, alkyl C fractions dramatically decreased at 400°C and disappeared when litter was heated at 500°C for 30 minutes.

The aromatic and phenolic C fractions (from 111 to 160 ppm), typical of charred plant residues (Knicker, 2007), progressively increased with heating temperatures. However, at 300°C a significant increase of this fraction was clearly detectable only for herbaceous materials such as *Eruca* and *Medicago*, for which we found a corresponding decrease of O-alkyl C. This observation supports the hypothesis that aromatic C compounds do not reflect only a selective, relative enrichment deriving from the removal of other compounds, but also a thermal neo-formation that likely involves previously dehydrated carbohydrates (González-Pérez et al., 2004). A similar process may drive to a massive production of the aromatic and phenolic fraction that dominate the ^{13}C CPAS NMR spectra of litter heated at 400°C and 500°C.

3.5.2. Organic matter preference by plant and microbes

We found that all undecomposed litter caused a severe inhibition of *Lepidium* root growth. The inhibitory effect by undecomposed litter found for eight litter types is in agreement with previous studies carried out in both natural and agro-ecosystems (Dorrepaaal et al., 2007; Bonanomi et al., 2011a; Lopez-Iglesias et al., 2014; Meiners, 2014; Mazzoleni et al., 2015). Our results confirm that this effect is a rather general phenomenon not restricted to a few “allelopathic” plants. However, inhibition by plant litter largely decreased (i.e. *Pinus*, *Hedera* and to a lesser extend for *Medicago*) or even disappeared (i.e. *Eruca* and *Quercus*) after heating at 400°C and 500°C.

Fig. 3. Responses of plant and microbial species exposed to watery extracts (50 g l⁻¹) of plant litters either untreated (NT) or heated at five different temperature levels for 30 minutes. Data refer to plant root growth (A), hyphal growth (B, D, F) and spore germination (C, E, G) and are expressed as percentage of unexposed controls. Values are average of 10 replicates, error bars are omitted to improve readability (statistical analysis in Supplementary Tables S3 and S4).



To explain why undecomposed litter is broadly inhibitory on root growth and why this harmful effect largely decreases as litter is heated at temperatures higher than 300°C, first we have to take into account that extract preparation from this litter basically consists of cell contents released by plant tissue breakdown. At this stage, litter with herbaceous, lignin poor materials (i.e. *Medicago*, *Hedera* and *Eruca*) were more inhibitory on *Lepidium* root growth compared with more lignified materials (e.g. *Pinus* and *Quercus*). The inhibitory effect of root growth by undecomposed plant litter has been either related to litter N immobilization (Hodge et al., 2000) or to the phytotoxic activity by allelopathic factors (Bonanomi et al., 2011a). According to the nutrient immobilization hypothesis, litter with high C/N ratio, by reducing the available nitrogen, limits root system development. Our results contradict this hypothesis because the inhibitory effect was stronger for species with lower C/N ratio (e.g. *Eruca*, *Hedera* and especially *Medicago* litter). This indicates that, at least for the selected species and under laboratory conditions, root growth is not inhibited by mineral N starvation over undecomposed litter. Probably, other factors are involved in the general inhibitory effects of undecomposed plant litter, although N limitation may be important in specific ecological conditions, e.g. litter with high C/N ratio and in N poor soils (Michelsen et al., 1999; Bowman et al., 2004). Concerning the alternative hypothesis that undecomposed plant litter can release allelopathic compounds with harmful effects on root growth (Rice, 1984), we found that *Lepidium* root growth was negatively correlated with the methoxyl C and especially O-alkyl C regions of ^{13}C CPMAS NMR spectra. This result is consistent with a previous study carried out with plant litter decomposed in soil microcosms (Bonanomi et al., 2011a) and indicates that poorly lignified plant materials have inhibitory effects on root proliferation. On the other hand, root growth was positively associated to the two aromatic regions of ^{13}C NMR spectra (Table 1). The extensive correlation analysis applied along the whole ^{13}C NMR spectra also showed a consistent positive association of aromatic C types with root growth. In fact, the entire H- and C-substituted aromatic C region (111-140 ppm) in general was positively correlated to *Lepidium* root growth. The restricted section (120-130 ppm), corresponding to peaks typical of charred materials (Knicker, 2007), displayed the highest positive correlation (Fig. 4). A number of previous studies related the inhibitory, allelopathic effect of plant residues to aromatic compounds (e.g. Rice, 1984; Blum et al., 1999). Our results, instead, indicate that aromatic and aryl C compounds do not inhibit or, even promote plant growth. A further explanation for the plant growth promoted by charred litter can be related to its well-known capability to adsorb and neutralize phytotoxic organic molecules with a limited impact on mineral nutrients (Zackrisson et al., 1996; Hille & den

Ouden, 2005). In general, our findings are in agreement with the growing body of literature showing the positive effect of biochar, in different forms and uses, as amendment in various agricultural systems (Jeffery et al., 2011). Future works are needed to confirm and validate the generality of our data by using a wider spectra of target species. In addition, further studies are required to clarify the molecular and physiological basis of the positive effects of aromatic and phenolic fractions on plant growth.

Table 1 Simple correlation (Pearson's r) between ^{13}C -CPMAS NMR data describing biochemical quality of 30 litter types and root growth of *Lepidium sativum*, spore germination of three fungi (*Aspergillus niger*, *Penicillium italicum*, and *Trichoderma harzianum*), and hyphal growth of two fungi (*Ganoderma lucidum* and *Rhizoctonia solani*) and an oomycete (*Pythium ultimum*) grown on the litter materials. Bold indicates statistical significant r values ($P < 0.0071$, after correction for multiple comparisons according to the Bonferroni's method).

Litter biochemical quality	Target organism						
	<i>Lepidium</i>	<i>Aspergillus</i>	<i>Ganoderma</i>	<i>Penicillium</i>	<i>Pythium</i>	<i>Rhizoctonia</i>	<i>Trichoderma</i>
Carboxylic C: 161-190 ppm	-0.26	0.52	0.40	0.41	0.71	0.75	0.50
O-sub. aromatic C : 141-160 ppm	0.65	-0.80	-0.73	-0.71	-0.64	-0.60	-0.80
H- & C-sub. aromatic C: 111-140 ppm	0.69	-0.81	-0.74	-0.72	-0.54	-0.50	-0.82
di-O-alkyl C: 91-110 ppm	-0.41	0.39	0.42	0.43	0.09	0.01	0.43
O-alkyl C: 61-90 ppm	-0.69	0.85	0.81	0.79	0.54	0.51	0.86
Methoxyl and N-alkyl C: 46-60 ppm	-0.57	0.78	0.65	0.68	0.70	0.71	0.77
Alkyl C: 0-45 ppm	-0.31	0.25	0.20	0.18	0.34	0.29	0.23

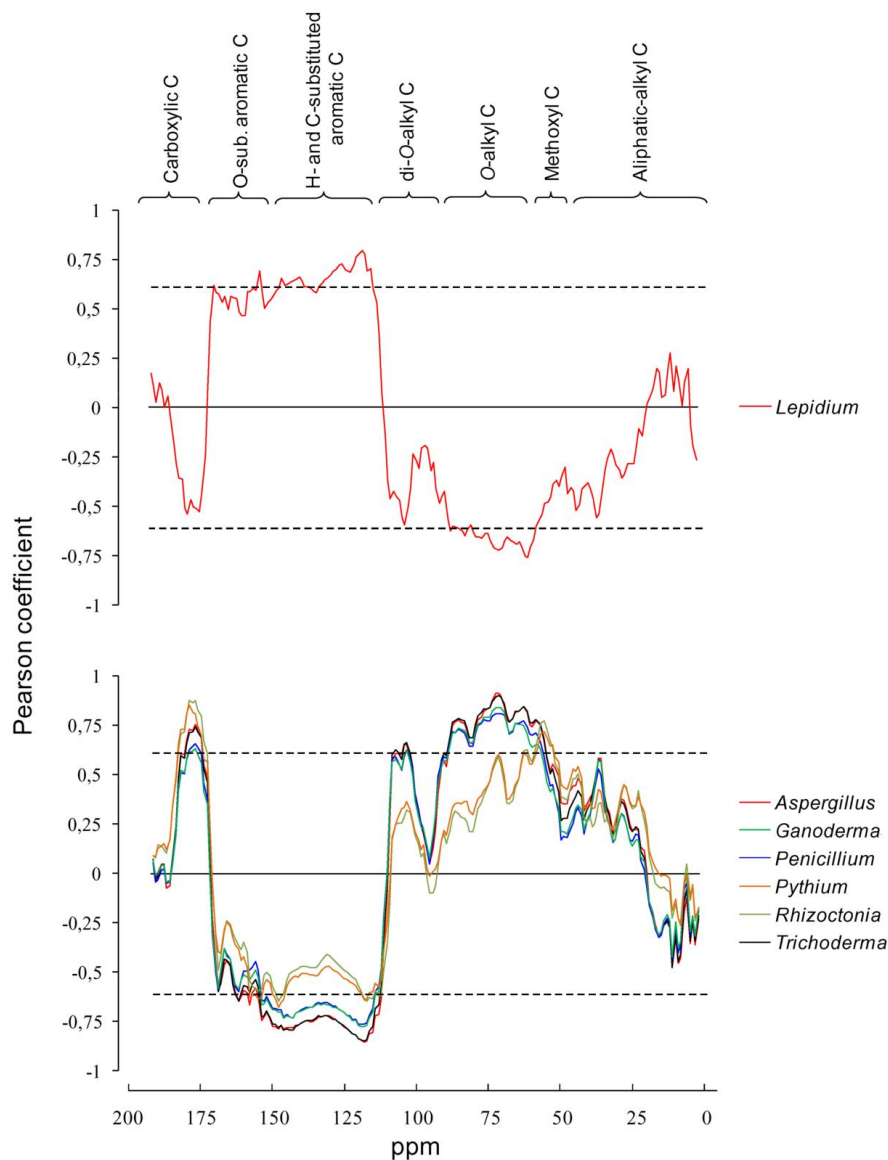
In our experiments, the fungi and oomycete thrive on most water extracts of undecomposed litter, with growth rate remarkably higher, similar or slightly lower than that recorded for the controls, over rich, standard microbiological substrates (PDA). However, a certain variability exists among litter types, with microbes preferring fast decomposing, herbaceous, nitrogen-fixing or lignin poor litter (i.e. *Eruca*, *Medicago*, and *Hedera*), rather

than highly lignified litter (i.e. *Pinus* and *Quercus*). These results are consistent with those by Bonanomi et al. (2011b) and Incerti et al. (2013) reporting a high growth rate of 18 fungal species over several undecomposed leaf litter. Microbial inhibition was observed only with soilborne pathogens (i.e. *Pythium* and *Rhizoctonia*) over lignified, tannin rich litter of *Pinus* and *Quercus*. Such microbes are reported to be aggressive saprophytes, especially on fresh, herbaceous plant residues, mostly green manure (Rothrock and Kirkpatrick, 1995; Manici et al., 2004). However, their ability to colonize lignin and tannin rich materials is quite limited (Erhart et al., 1999), likely because of their limited enzymatic arsenal (Sneh et. al., 1996) compared with saprophytic microbes.

In contrast to the higher plant, all tested microbes showed either a steep decline or a complete growth inhibition over litter heated at temperatures $\geq 300^{\circ}\text{C}$. Such a finding could be explained by the temperature-dependent reduction of litter biochemical quality, which makes the organic materials unsuitable for microbial exploitation. Changes of litter suitability as a substrate for microbial growth could be related to: (i) a decrease of easily degradable C sources; (ii) an accumulation of toxic and/or aromatic organic compounds. In this respect, ^{13}C NMR data clearly show, as litter is progressively heated at increasing temperatures, a sharp decrease of the labile C fraction and a relative increase of aromatic, char typical C compounds. In detail, ^{13}C -CPMAS NMR analysis of peat (Almendros et al., 2003) and grass residues (Knicker et al., 2005) reported a rapid temperature-dependent reduction of carbohydrates (spectral regions at 61-110 ppm, corresponding to di-O-alkyl C and O-alkyl C). This supports the hypothesis that microbial growth inhibition over heated litter is controlled by the availability of easily degradable carbon sources. On the other hand, the possibility exists that newly formed aromatic and phenolic compounds, typical products of charred plant residues, may directly inhibit microbial growth. In our experiments, however, the addition of simple C sources (e.g. glucose, unpublished results) to watery extracts of heated litters restored microbial growth in most cases, thus supporting the C starvation hypothesis. In conclusion, our findings suggest that the effect of undecomposed litter and organic material heated at different temperatures on microbes can be the result of the balance between the availability of labile organic C sources and the presence of recalcitrant and/or fungitoxic compounds that provide little support or even inhibit microbial growth. Finally, it is noteworthy that the six tested microbes showed a remarkably similar pattern of correlation between growth and litter quality, as defined by ^{13}C CPMAS NMR. An opposite response was evidenced by the higher plant *Lepidium*, in terms of both litter quality and its correlation with root growth. These new results highlight the need for further work aimed to clarify the

relationships between the biochemical quality of organic matter and the multiple effects on different ecosystem trophic levels.

Fig. 4. Profiles of correlation (Pearson's r) between species responses to litter extracts and ^{13}C -CPMAS NMR spectral signals for the same materials. Species responses related to spectral data are root growth of *Lepidium sativum* (above), and spore germination or hyphal growth of five fungi and an oomycete (below). Species response and spectral data refer to litter extracts at high concentration (50 g l^{-1}) from 5 litter types heated at 6 different temperatures ($N=30$). Dashed lines indicate threshold values of statistical significance for r ($P < 0.00025$, after correction for multiple comparisons according to the Bonferroni's method). The seven main classes of organic C assessed by ^{13}C -CPMAS NMR spectroscopy for the 30 organic materials are reported on top of the panels.



3.6 CONCLUSION

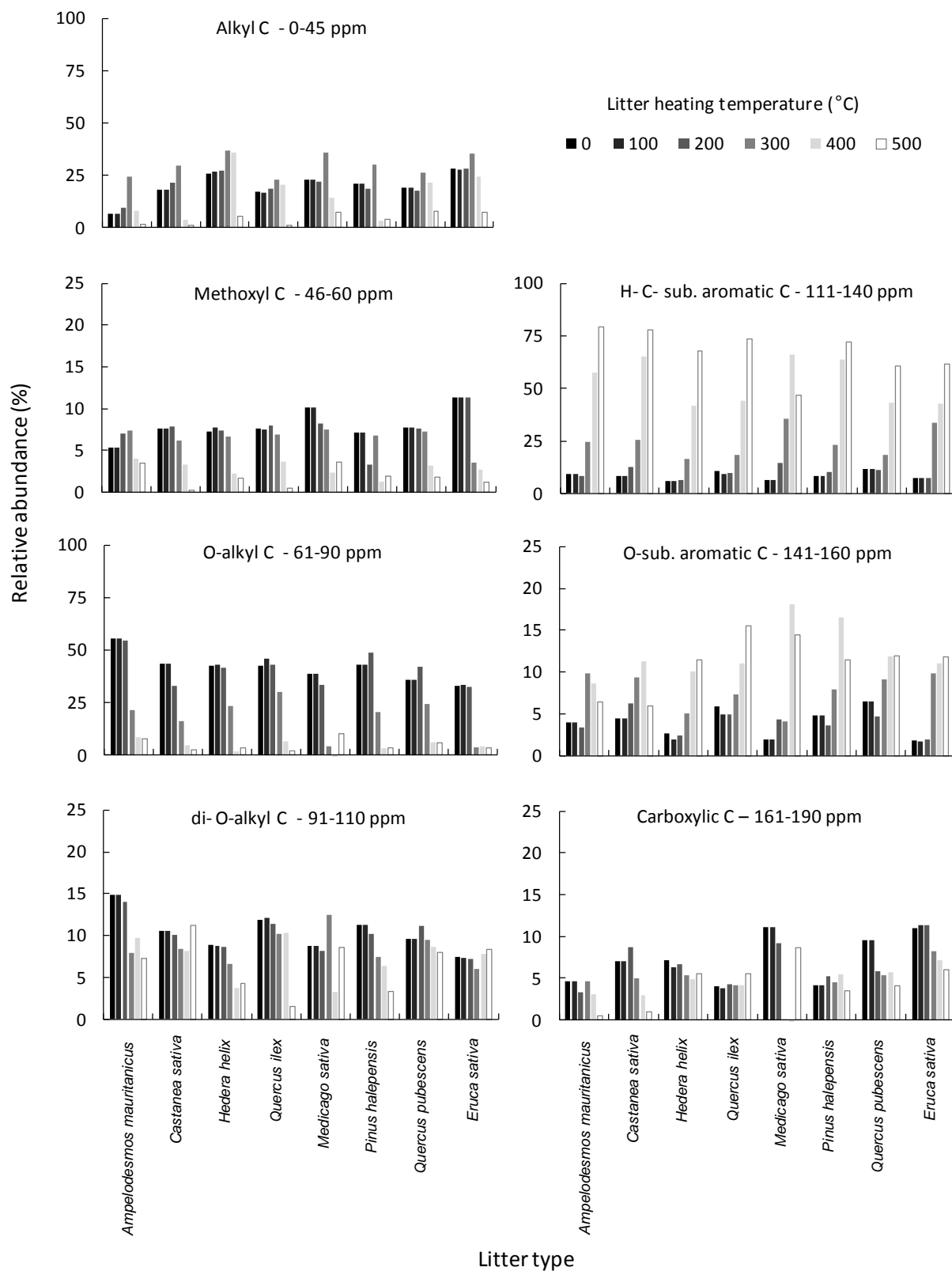
In this work, we provided empirical evidence that improve our understanding of the substrate preferences by plants and microbes for organic substrates. The use of ^{13}C -CPMAS NMR allowed a detailed monitoring of the chemical changes occurring in litters during the charring treatment. In agreement with previous studies (Knicker, 2007), we found consistent and progressive losses of Oalkyl C, di-O-alkyl C as well as methoxyl and N-alkyl C, for all types of plant litters, with corresponding increases of aromatic C. Noteworthy, we demonstrated that undecomposed litters have a major inhibitory effect on the test plant, while acting as a substrate to sustain microbial growth. On the contrary, as litter is charred at high temperatures its biochemical quality decreases because of a progressive disappearance of easily degradable C sources and enrichment of aromatic fractions. Therefore, after charring litter becomes an organic material suitable to sustain plant growth, but largely inhibitory for fungal and oomycete performances. We are aware that the implications of our findings for the understanding of the relationships between litter C quality and the multiple effects on different ecosystem trophic levels is limited by the use of few species studied by short-term laboratory bioassay. Then, future studies are required to evaluate the present findings in more realistic ecological conditions.

ACKNOWLEDGMENTS

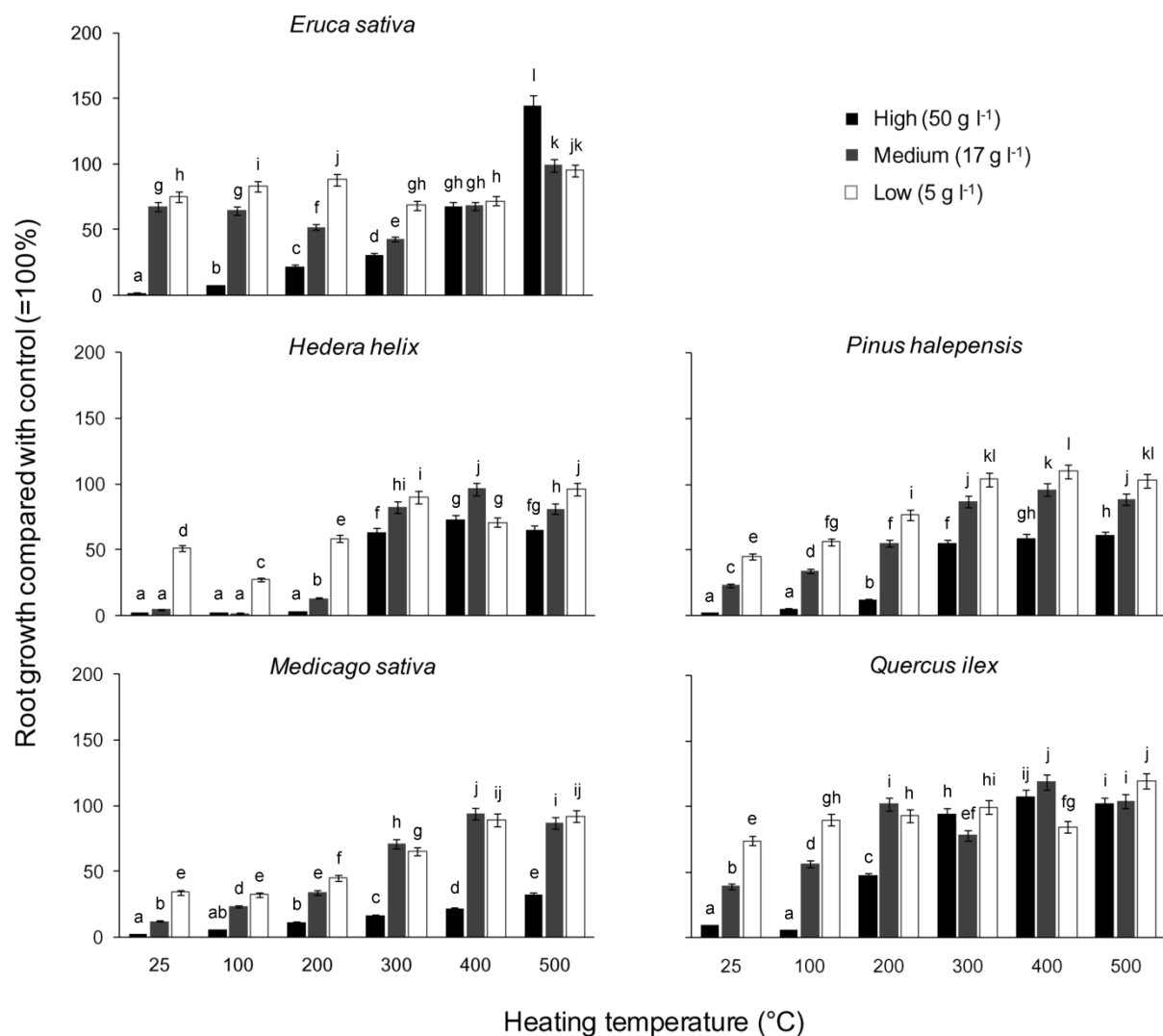
The ^{13}C -CPMAS NMR measurements were performed at the CERMNU-Interdepartmental Research Centre, University of Naples Federico II.

3.7 SUPPLEMENTARY TABLES AND FIGURES

Supplementary Fig. S1. Variation of seven main classes of organic C assessed by ^{13}C -CPMAS NMR spectroscopy in each of eight plant litters treated at six different temperatures for 30 minutes. Note different scales on y-axes.



Supplementary Fig. S2. Results of the “root proliferation” bioassay: *Lepidium sativum* root growth, compared to control (=100%) into each different litter either untreated or heated at five temperature for 30 minutes. Watery extract of each organic material was used at three concentrations (High = 50 g l⁻¹; Medium = 17 g l⁻¹; Low = 5 g l⁻¹). Values are averages ± SD of 3 replicates for each litter types. Within each litter type, different letters indicate statistically significant pair-wise differences for the interactive effect of litter heating temperature and extract concentration (Tukey's HSD post-hoc test from GLM in Supplementary Table S2, *P* < 0.05).



Supplementary Table S1. C/N ratio, nitrogen and lignin content of the eight litter types. Values are the average of three replicates \pm standard deviation. Different letters within each row indicate statistically significant differences (One-way ANOVA, $P < 0.05$). Lignin content is expressed as ash free relative value of total structure.

	N content (%)	C/N ratio	Lignin content (%)
<i>Ampelodesmos mauritanicus</i>	1.19 \pm 0.23	41.32 \pm 3.23	11.20 \pm 2.14
<i>Castanea sativa</i>	2.17 \pm 0.88	24.21 \pm 3.12	13.52 \pm 1.95
<i>Eruca sativa</i>	3.72 \pm 0.43	11.25 \pm 1.32	2.31 \pm 0.87
<i>Hedera helix</i>	2.02 \pm 0.12	21.86 \pm 2.11	5.78 \pm 0.76
<i>Medicago sativa</i>	3.91 \pm 0.48	9.12 \pm 2.1	6.77 \pm 2.23
<i>Pinus halepensis</i>	1.32 \pm 0.87	37.53 \pm 3.4	17.31 \pm 2.39
<i>Quercus ilex</i>	1.24 \pm 0.21	31.72 \pm 3.43	18.36 \pm 2.91
<i>Quercus pubescens</i>	1.65 \pm 0.72	32.51 \pm 4.1	21.72 \pm 3.71

Supplementary Table S2. List of fungi and oomycete used in this study comprising soilborne and airborne pathogens, saprophytes and antagonistic microbes.

Species	Phylum	Functional group
<i>Aspergillus niger</i>	Ascomycota	Saprophyte, opportunistic plant pathogen
<i>Ganoderma lucidum</i>	Basidiomycota	Saprophyte, plant pathogen
<i>Penicillium italicum</i>	Ascomycota	Plant pathogen
<i>Pythium ultimum</i>	Heterokontophyta	Soilborne plant pathogen
<i>Rhizoctonia solani</i>	Basidiomycota	Soilborne plant pathogen
<i>Trichoderma harzianum</i>	Ascomycota	Saprophyte, mycoparasite

Supplementary Table S3. Statistical support to *Lepidium sativum* bioassay. Summary of the GLM testing for main and interactive effects of litter type, heating temperature, and watery extract concentration on *Lepidium sativum* root growth, expressed as percentage of the control treated with distilled water.

Source of variation	SS	df	MS	F	p
Litter type (L)	12647.8	4	3161.9	15.1	< 0.00001
Heating temperature (T)	60391.6	1	60391.6	289.0	< 0.00001
Extract concentration (C)	38628.4	2	19314.2	92.4	< 0.00001
L × T	259567.0	4	6489.2	31.1	< 0.00001
L × C	4011.9	8	501.5	2.4	0.01657
T × C	3139.5	2	1569.8	7.5	0.00068
L × T × C	10853.5	8	1356.7	6.5	< 0.00001

Supplementary Table S4. Statistical support to microbial bioassays. Summary of the GLM testing for main and interactive effects of litter type, heat treatment, and extract concentration on spore germination (a, c, f) and hyphal growth (b, d, e) of six microbial species, expressed as percentage of the controls. In a further GLM (g), data were pooled for all species and main and interactive effects of microbial species and litter treatments were also included.

Source of variation	SS	df	MS	F	p
a) <i>Aspergillus niger</i>					
Litter type (L)	16268.6	4	4067.1	11.1	< 0.00001
(T) Heating temperature	283587.9	1	283587.9	771.2	< 0.00001
(C) Extract concentration	79820.7	1	79820.7	217.1	< 0.00001
L × T	7995.9	4	1999.0	5.4	0.00039
L × C	2187.0	4	546.7	1.5	0.20859
T × C	37047.4	1	37047.4	100.8	< 0.00001
L × T × C	964.7	4	241.2	0.7	0.62355
b) <i>Ganoderma lucidum</i>					
Litter type (L)	8578.1	4	2144.5	2.9	0.02418

(T)	Heating temperature	240213.0	1	240213.0	323.4	< 0.00001
(C)	Extract concentration	40967.4	1	40967.4	55.2	< 0.00001
	L × T	7695.9	4	1924.0	2.6	0.03873
	L × C	28789.4	4	7197.4	9.7	< 0.00001
	T × C	34972.6	1	34972.6	47.1	< 0.00001
	L × T × C	16818.4	4	4204.6	5.7	0.00027

c) *Penicillium italicum*

	Litter type (L)	20287.8	4	5072.0	7.0	0.00003
(T)	Heating temperature	369711.2	1	369711.2	509.0	< 0.00001
(C)	Extract concentration	92802.2	1	92802.2	127.8	< 0.00001
	L × T	10063.6	4	2515.9	3.5	0.00961
	L × C	5592.6	4	1398.1	1.9	0.10883
	T × C	44252.5	1	44252.5	60.9	< 0.00001
	L × T × C	1269.2	4	317.3	0.4	0.78189

d) *Pythium ultimum*

	Litter type (L)	71080.2	4	17770.0	89.5	< 0.00001
(T)	Heating temperature	67621.8	1	67621.8	340.6	< 0.00001
(C)	Extract concentration	18562.4	1	18562.4	93.5	< 0.00001
	L × T	30016.5	4	7504.1	37.8	< 0.00001
	L × C	23759.2	4	5939.8	29.9	< 0.00001
	T × C	10263.1	1	10263.1	51.7	< 0.00001
	L × T × C	12678.8	4	3169.7	16.0	< 0.00001

e) *Rhizoctonia solani*

	Litter type (L)	227732.4	4	56933.1	60.3	< 0.00001
(T)	Heating temperature	161902.6	1	161902.6	171.4	< 0.00001

(C)	Extract concentration	42413.2	1	42413.2	44.9	< 0.00001
	L × T	107232.6	4	26808.2	28.4	< 0.00001
	L × C	44343.0	4	11085.8	11.7	< 0.00001
	T × C	35305.4	1	35305.4	37.4	< 0.00001
	L × T × C	22679.6	4	5669.9	6.0	0.00016
f) <i>Trichoderma harzianum</i>						
(T)	Litter type (L)	21879.9	4	5470.0	9.8	< 0.00001
	Heating temperature	452802.8	1	452802.8	809.0	< 0.00001
	Extract concentration	78040.3	1	78040.3	139.4	< 0.00001
	L × T	5336.0	4	1334.0	2.4	0.05362
	L × C	1813.0	4	453.2	0.8	0.52060
(C)	T × C	38613.5	1	38613.5	69.0	< 0.00001
	L × T × C	1288.0	4	322.0	0.6	0.68093
g) All species						
(T)	Microbial species (S)	66005	5	13201.2	12.2	< 0.00001
	Litter type (L)	117998	4	29499.4	27.3	< 0.00001
	Heating temperature	1466196	1	1466196.3	1357.7	< 0.00001
	Extract concentration	145154	1	145154.5	134.4	< 0.00001
	S × L	162515	20	8126.3	7.5	< 0.00001
(C)	S × C	26958	5	5391.8	5.0	0.00016
	L × C	19794	4	4947.8	4.6	0.00114
	S × L × C	45868	20	2293.1	2.1	0.00278

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Chapter 4

Effects of organic feedstocks and their biochars on growth of phytopatogenic fungi, soilborne bacteria, and crop plants

4. EFFECTS OF ORGANIC FEEDSTOCKS AND THEIR BIOCHARS ON GROWTH OF PHYTOPATOGENIC FUNGI, SOILBORNE BACTERIA, AND CROP PLANTS

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4.1 ABSTRACT

A number of recent works show that biochar significantly reduces the incidence of plant diseases caused by airborne and soilborne pathogens.

In this context, we investigated how biochar affects plant growth and microbial activity.

The aims of this study were i) to detect the chemical changes occurring on different feedstocks when pyrolyzed at two specific temperatures (300 °C and 550 °C) by using ¹³C-CPMAS NMR spectroscopy and ii) to assess how biochar can influence the growth of fungi, bacteria, and crop plants. In particular, 12 different organic materials, including pyrolyzed and untreated materials, were used. The experimental design compared the effect of amendments carried out by using biomasses untreated and the derived biochars. We found that undecomposed organic materials may cause a severe inhibition of plant root growth but, in some cases, have a stimulating effect. However, the inhibitory effect largely decreased after pyrolyzation. In contrast to the higher plants, fungi and bacteria thrived on most of the organic materials and showed a steep decline or a complete growth inhibition on biochars obtained at 300°C and 550°C. One of the most significant pieces of evidence from this study is that twelve out of fourteen tested microbes, with the exception of two basidiomycetes, showed a remarkably similar pattern of correlation between growth and organic material quality, as defined by ¹³C CPMAS NMR. An opposite response was observed for the higher plants *Lepidium*, *Lactuca* and *Lycopersicon* in terms of both chemical quality and its correlation with root growth.

4.2 INTRODUCTION

The last decades have seen a growing trend towards the study of biochar. This product is obtained when biomass is heated at high temperature in a low-oxygen environment (Lehmann 2009). The positive effect of biochar to improve the fertility of soil originated in the Amazon since ancient times (Steiner et al., 2004). For its demonstrated capability in increasing crops production (Jeffery et al., 2011) the biochar is largely used as a soil amendment, and commercialized throughout the world (Morell et al., 2013).

Currently, the interest of some researchers is moving towards the use of biochar also as a biocontrol agent of plant diseases (Bonanomi et al., 2015). A number of recent works (Harel et al., 2012; Elmer and Pignatiello, 2011; Graber et al., 2014) show that biochar significantly reduces the incidence of plant diseases caused by airborne and soilborne pathogens. Some of these studies attribute to biochar an ability to induce systemic resistance and a consequent reduction in the incidence of diseases (Elad et al., 2010; Harel et al., 2012; Zwart and Kim 2012; Mehari et al., 2015), while others found that the biochar acts as a carrier for biological control agents (Postma et al., 2013). However, in most cases the suppressive mechanisms are not clearly identified so far (Jaiswal et al., 2014a,b).

Several studies by using also analytical techniques such as pyrolysis-gas chromatography/mass spectrometry (Nocentini et al., 2010) and ^{13}C -CPMAS nuclear magnetic resonance (NMR) spectroscopy (Kögel-Knabner, 2002; Almendros et al., 2003) revealed that substantial changes occur above 270°C. Above this threshold, organic material rapidly loses nutrients (Gray and Dighton, 2006), decreases the content of carbohydrates and proteins and becomes progressively enriched in aromatic organic carbon compounds (review in Knicker, 2007). The composition of biochar depends on the starting feedstocks, the pyrolysis conditions including time, and temperature (Lehmann et al., 2009). Recently, Bonanomi et al., (2016) demonstrated that heat treatment of organic materials determines some chemical changes. Few studies have shown that chemical changes can affect growth of plants and microorganisms. Also in this context, we investigated how biochar affect plant growth and microbial activity and we selected four organic materials to represent a wide range of organic matter quality. We chose four feedstocks that were pyrolyzed at two specific temperatures (300°C and 550°C) to obtain eight biochars plus four untreated organic materials. Those 12 materials that we produced have been tested on three plants, nine fungi (saprotrophic, pathogens, and biocontrol agents) and five bacteria (pathogens, and biocontrol agents). Most notably, only one study reported an experimental design that compared the effect of amendment by biomass untreated and the derived biochars (Calderon et al., 2015),

but this study is focused only on C sequestration biochar capability and plant growth compared to the same untreated material (*Zea mays*). Other works focused their attention only on biochar and microbes leaving out plant growth on the same materials. We studied also relationships between chemical composition of organic materials and biochar, defined by ^{13}C NMR spectroscopy, with growth of the fungi, bacteria, and plants.

In this study we combined organic material and biochar characterization by ^{13}C -cross-polarization magic angle spinning (CPMAS) nuclear magnetic resonance (NMR) spectroscopy (Kögel-Knabner, 2002), with a multi-species bioassay approach.

Specific aims of the study were:

- (1) To detect the chemical changes occurring on different feedstocks when heated at two specific temperature (300 °C and 550 °C) by using ^{13}C -CPMAS NMR spectroscopy;
- (2) To assess the effect of biochar on the growth of fungi, bacteria, and plants;
- (3) To explore the relationships between biochar chemistry, defined by ^{13}C -CPMAS NMR spectroscopy and growth of target species.

4.3 MATERIALS AND METHODS

4.3.1 Organic feedstock and biochar production

Four different feedstocks of organic materials were selected to represent a wide range of organic matter chemical quality: F.O.R.S.U., (Organic Fraction Solid Municipal Waste), wood chips; *Medicago sativa* hay, and *Zea mays* stalks. Eight types of biochar were prepared from the four feedstocks, each at a treatment temperature of 300 and 550 °C, and were used throughout the research. All biochars were heated in a muffle furnace for 5 hours. Then, the material was ground by a mixer to obtain a powder of <5 mm particles and stored in sealed containers.

To obtain biochar and organic material water extracts for bioassays, dried materials were mixed with distilled water in a beaker at 5% of dry weight (50 g l⁻¹) and shaken for 5 h. The aqueous suspensions were then centrifuged (2395 g for 10 min), sterilized (microfiltration with 0.22-μm pore filter) and stored at -20°C until bioassay.

4.3.2 Plant bioassay

The bioassays were aimed at assessing the effects of the four organic untreated materials untreated and the eight biochars on the growth of three plant species. The plant bioassay,

hereafter called the “seed germination” experiment, was carried out on *L. sativum* as a target species because of its recognized sensitivity to phytotoxic compounds (Bonanomi et al., 2011a), and on other two species chosen for their role in horticulture and economical importance: *Lactuca sativa* and *Lycopersicon esculentum*.

Briefly, 25 seeds of each species were placed in 9 cm Petri dishes over sterile filter papers with 4 ml of test solution. The water extract were diluted by distilled water to three concentrations (0, 10, 33 %) and stored at -20°C until bioassay. Every solution plus the control with distilled water were replicated 3 times for a total of 2.700 (25 seeds x 3 concentrations x 12 extracts x 3 replicates) seeds for the whole experiment. Petri dishes were arranged in a growing room in a completely randomized design and seedling root length was measured 36 hours after germination for *Lepidium* and after five days for *Lactuca* and *Lycopersicon*.

4.3.3 Fungal and bacterial bioassay

The microbial bioassay was performed to assess the effects of organic materials on the saprotrophic growth of nine fungi with different functional features: *Aspergillus niger* (saprotrophic), *Botrytis cinerea*, *Fusarium oxysporum*, *Fusarium oxysporum* f. sp. *radicis lycopersici*, *Ganoderma lucidum*, *Penicillium italicum*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum* (pathogens), and *Trichoderma harzianum* (biocontrol agent). All strains were available at the mycology laboratory of the Department of Agriculture, University of Naples, and maintained on potato dextrose agar (PDA, Oxoid) medium. For six fungi (*A. niger*, *B. cinerea*, *P. italicum*, two species of *Fusarium* and *T. harzianum*) we used a spore germination bioassay. The inoculum was obtained by adding 10 ml of sterile water to ten- day old cultures, grown in Petri dishes containing PDA, and by scraping the culture surface to remove conidia. The suspension was filtered, centrifuged, washed twice with sterile water and adjusted to a concentration of 10^5 conidia ml^{-1} by haemocytometer counting chamber. Spore suspension was prepared in 10 μl of sterile water. Organic materials extracts were applied in 96-well plates and incubated at 24°C . Fungal growth was measured spectrophotometrically ($\lambda = 590 \text{ nm}$) after 20 hours of incubation using a Thermomax microtitre (Thermomax microtiter) plate reader (Molecular Devices, Wokingham, UK). For *G. lucidum*, *R. solani* and *S. sclerotiorum* which do not produce conidia, hyphal growth bioassay was performed.

Since the aim of this experiment was to test the ability of the microbes to use organic material and biochar as the single source of nutrient, microbial growth was started from a

water agar (WA, Oxoid) nutrient-poor medium. After seven days of culture on WA, a 4 mm diameter plug was collected from the edge of the growing colony and placed in the centre of the Petri dish. For such microbes substrates were prepared by mixing WA and sterile litter extracts at 1:1 ratio. Ten millilitres of each dilution were placed in a 9 cm Petri dish. PDA was used as the control substrate. Ten replicates were used for each treatment. After 72 h, hyphal density and radial growth of each colony were measured on five randomly chosen points. Hyphal density was measured by counting the number of hyphae crossing a 1 mm line at 250× magnification under binocular microscope. A growth index was calculated as the product of the area of fungal colony, calculated from the measured colony radius, and the hyphal density, following Tuitert et al., (1998).

The bacterial assay was performed to assess the effects of organic materials on the saprotrophic growth of five bacteria species: three biocontrol agents, *Agrobacterium tumefaciens* K84, *Lysobacter* sp., *Bacillus subtilis*, and two pathogens, *Pseudomonas viridiflava* and *Pseudomonas syringae* pv. tomato. All the colonies were available at the bacteriology laboratory of Department of Agriculture, university of Naples, and maintained in 30% glycerol. The inoculum was obtained by adding 5 µl of bacterial solution to a concentration of 10^9 ml⁻¹, grown in glass tube with LB broth. Organic materials and biochar extracts were applied in 96-well plates and incubated at 24°C. Bacterial growth was measured spectrophotometrically (λ = 590 nm) after 48 hours of incubation using a Thermomax microtitre plate reader (Molecular Devices, Wokingham, UK).

4.3.4 ¹³C-CPMAS NMR characterization and chemical analysis of organic feedstocks and biochar

The pH and electrical conductivity of the 12 organic samples (four untreated organic materials and eight biochars) were measured using a pH-meter (Basic 20 CRISON) and conductivity meter (CRISON) in 1:2.5 and 1:5 organic material: water suspensions, respectively. Organic material and biochars were characterized for total C, H and, N content by flash combustion of micro samples (5 mg of samples) in an Elemental Analyser NA 1500 (Fison 1108 Elemental Analyzer, Thermo Fisher Scientific).

All materials were characterized by ¹³C-CPMAS NMR obtained in solid state under the same conditions in order to perform a quantitative comparison among spectra. The spectrometer used was a Bruker AV-300 equipped with a 4 mm wide-bore MAS probe. NMR spectra were obtained with MAS of 13000 Hz of rotor spin, 1s of recycle time, 1 ms of

contact time, 20 ms of acquisition time, 2000 scans. Samples were packed in 4 mm zirconium rotors with Kel-F caps. The pulse sequence was applied with a ^1H ramp to account for non-homogeneity of the Hartmann-Hahn condition at high spin rotor rates. Each ^{13}C -CPMAS NMR spectrum was automatically integrated to calculate the area of the peaks which appeared in the chosen region. Spectral regions have been selected and corresponding C-types identified by previous studies (Kögel-Knabner, 2002; Bonanomi et al., 2011a): 0-45 ppm = alkyl C; 46-60 ppm = methoxyl and N-alkyl C; 61-90 ppm = O-alkyl C; 91-110 ppm = di-O-alkyl C; 111-140 ppm = H- and C- substituted aromatic C; 141-160 ppm O-substituted aromatic C (phenolic and O-aryl C); 161-190 ppm carboxyl C.

4.3.5 Data analysis

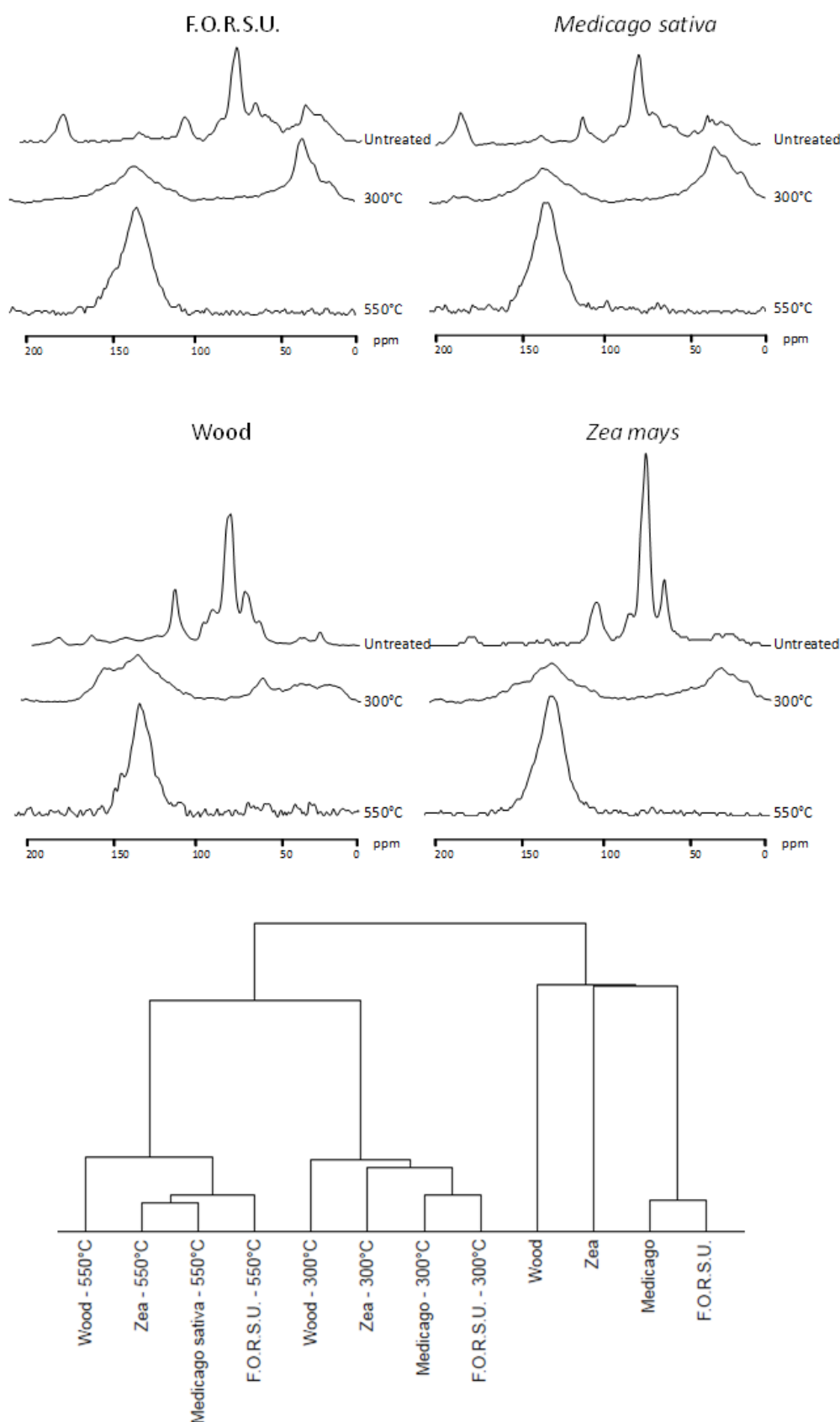
Two-way ANOVA was used to assess the effects of organic materials and pyrolyzation temperature on growth of all species. Then, we carried out 17 times, one for each species. Significance was evaluated in all cases at $P < 0.05$ and 0.01 .

To address the relationships between the performance of the plants, microbial species, and organic material and eight biochar chemistry (^{13}C -CPMAS NMR), linear correlation was calculated. Performance was tested for correlation with ^{13}C -CPMAS NMR spectral regions ($N = 7$) selected from reference literature (Almendros et al., 2000; Kögel-Knabner, 2002; Bonanomi et al., 2011a; Pane et al., 2011) and with each resonance signal ($N = 200$), providing a finer-resolution profile of the C-types variation in the tested materials associated with the effect on the growth performance of plants and microbes.

Moreover, simple linear correlation analysis was separately tested between plants and microbial species performance in the bioassays and organic materials and biochars chemical parameter (i.e. C content, N content, H content, C/N ratio, pH, and EC).

Cluster analysis, was carried out on ^{13}C -CPMAS NMR spectral regions of all organic materials and biochars to evaluate similarity among the samples. Finally, multivariate approach by using principal component analysis (PCA) was also performed. Following the approach suggested by Legendre and Legendre (1998) for supplementary variables, species growth, recorded in water extracts of biochars and organic materials, was also plotted as a loading vector on the bi-dimensional PCA space even if it was not used to compute the eigenvalues of the same ordination space.

Fig. 1. ^{13}C -CPMAS NMR spectra of F.O:R.S.U., wood chips, *Medicago sativa* hay and, *Zea mays* stalks organic materials and derived biochars pyrolyzed at two different temperature for 5 hours. Dendrogram of the cluster grouping of the organic materials and biochars.



4.4 RESULTS

4.4.1 Organic materials and biochar chemical changes associated with thermal treatments

Cluster analysis shows that the four untreated materials are similar with a clear difference with pyrolyzed biochars (Fig. 1). Moreover, two clusters were observed for material pyrolyzed at 300° and 550°C (Fig. 1).

The ^{13}C -CPMAS NMR spectra revealed significant and consistent differences of carbon types for all studied organic materials. Concerning untreated materials, two feedstocks, F.O.R.S.U. and *Medicago*, show an abundance of the aliphatic alkyl-C (0-45 ppm), and O-alkyl-C (91-110) (Fig. 1 and Fig.2). Instead, wood and *Zea* show an abundance of O-alkyl-C higher than the other two feedstocks. Among all organic materials, wood stands out for the presence of the H- and C- substituted aromatic C regions (111-140 ppm).

Four spectral intervals, corresponding to O-alkyl-C, di-O-alkyl-C, methoxyl and N-alkyl C as well as the carboxylic C region showed, a progressive decrease when organic materials were treated at 300°C. The relative decrease was especially marked for the O-alkyl-C (61-90 ppm), mainly associated with sugars and polysaccharides, that largely decreased at 300°C and almost disappeared when litter was treated at 550°C (Fig. 1 and Fig. 2). The aliphatic alkyl-C (0-45 ppm, characteristic of lipid waxes, cutins and microbial products) increases for all the feedstocks at 300°C, followed by a substantial decrease at 550°C (Fig. 1 and Fig. 2). Aromatic regions (111-140 ppm and 141-160 ppm), in contrast with other spectral regions, largely increased when organic materials were pyrolyzed at 300°C, and even more at 550°C (Fig. 1 and Fig. 2).

Tables 1 summarize the results of physical and chemical analysis of organic materials and biochars. With increasing thermal treatment, a progressive increase in the amount of C, pH and electrical conductivity for all the feedstock was observed. N content, instead, decreased for *Medicago* and increased for *Zea*, F.O.R.S.U. and wood with thermal treatment.

Table 1: Biochar and organic materials content of Carbon, Hydrogen, Nitrogen, pH, and electric conductivity.

	C %	N %	H %	C/N	pH	EC $\mu\text{s/cm}$
<i>Medicago sativa</i>	40,10	6,26	7,27	6,41	7,10	6430
<i>Medicago sativa</i> 300 °C	54,46	4,48	4,95	12,15	11,00	7530
<i>Medicago sativa</i> 550 °C	52,25	4,43	4,83	11,78	11,60	9490
<i>Zea mays</i>	40,38	0,7	7,80	57,68	6,54	2224
<i>Zea mays</i> 300 °C	67,54	2,48	2,17	27,24	9,13	2000
<i>Zea mays</i> 550 °C	68,66	2,46	4,24	27,95	10,86	3480
Wood	77,05	0,12	8,51	400	6,68	152,3
Wood 300 °C	85,59	0,13	3,54	639,04	7,77	138,6
Wood 550 °C	87,71	0,21	4,86	410,68	10,66	218
F.O.R.S.U.	51,08	4,77	-	10,71	6,84	4950
F.O.R.S.U. 300 °C	60,05	6,00	5,25	10,01	7,98	3840
F.O.R.S.U. 550 °C	61,25	6,26	5,32	9,78	11,41	10600

4.4.2 Biochar effects on plant growth

In the “seed germination” bioassay, feedstocks, temperature, and extract concentration, as well as their interactions, had a significant effect on plant growth (Table 2). At the highest extract concentration, *Zea* and wood had a stimulatory effect on *Lepidium* and *Lycopersicon* root growth independent of temperature treatment (Fig. 3). Instead, *Lactuca* root growth was stimulated only by wood feedstock and wood biochars at 300 °C and 550°C. The untreated F.O.R.S.U. and *Medicago* showed a remarkable inhibitory effect on all target species (Fig. 3). However, biochar from F.O.R.S.U. produced at 300°C displayed a clear stimulatory effect on *Lycopersicon* and *Lepidium*, while not significant effect was found for *Lactuca*. Finally, biochars produced from *Medicago*, displayed a reduced root growth compared to the untreated feedstock.

Fig. 2. Variation of relative abundance of seven main classes of organic C assessed by ^{13}C -CPMAS NMR spectroscopy in four organic materials and eight biochars pyrolyzed at two different temperatures for 5 hours.

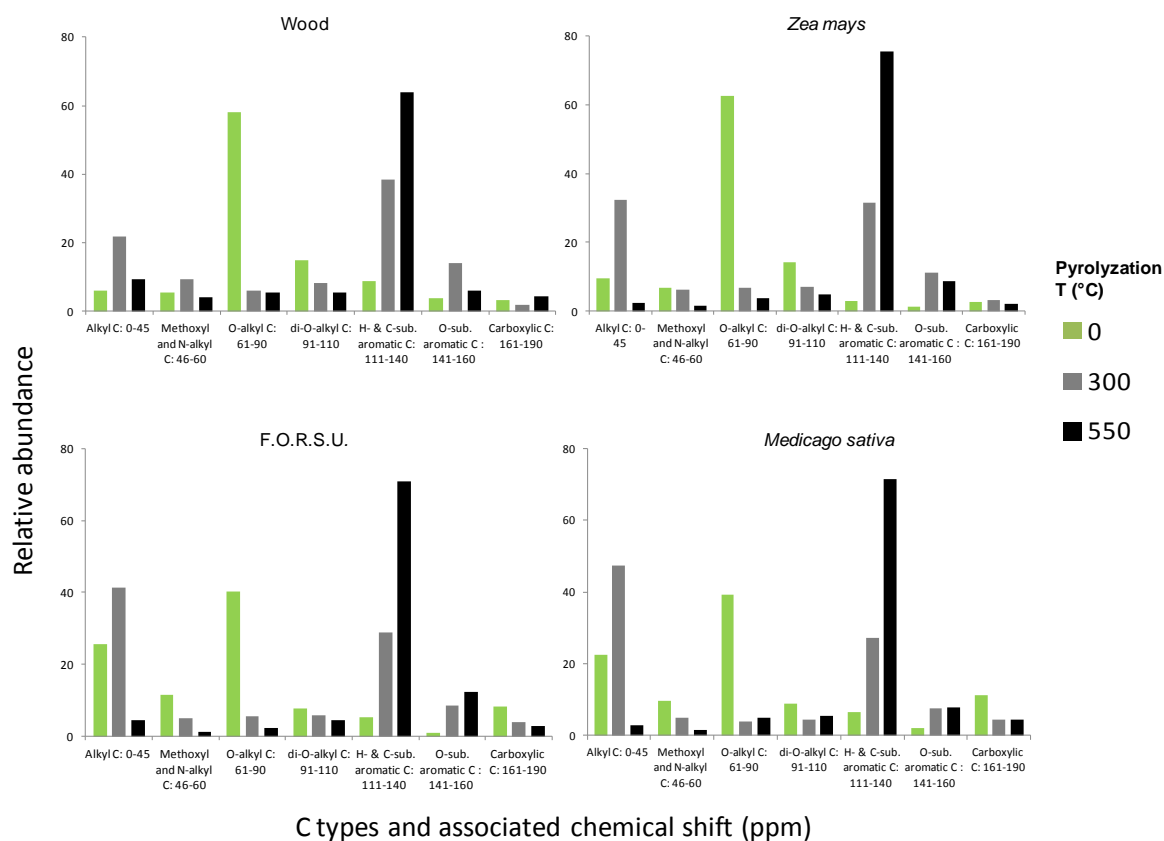
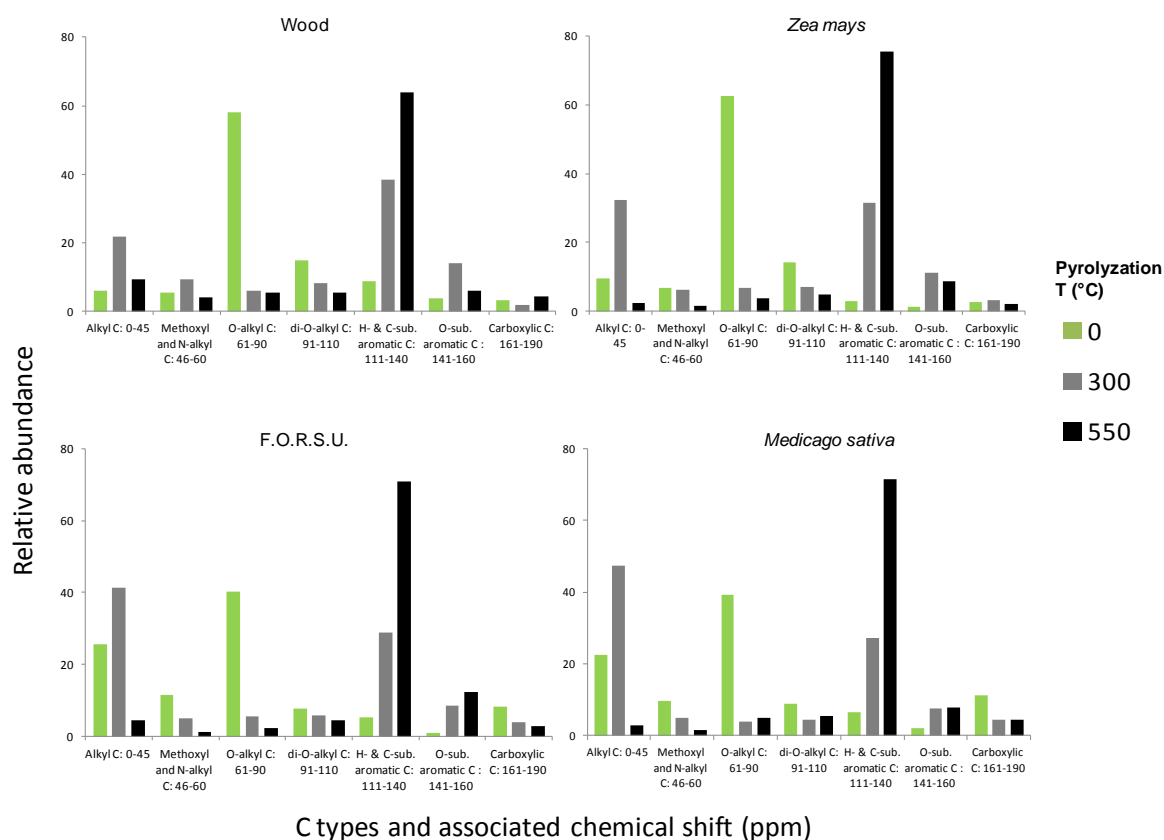


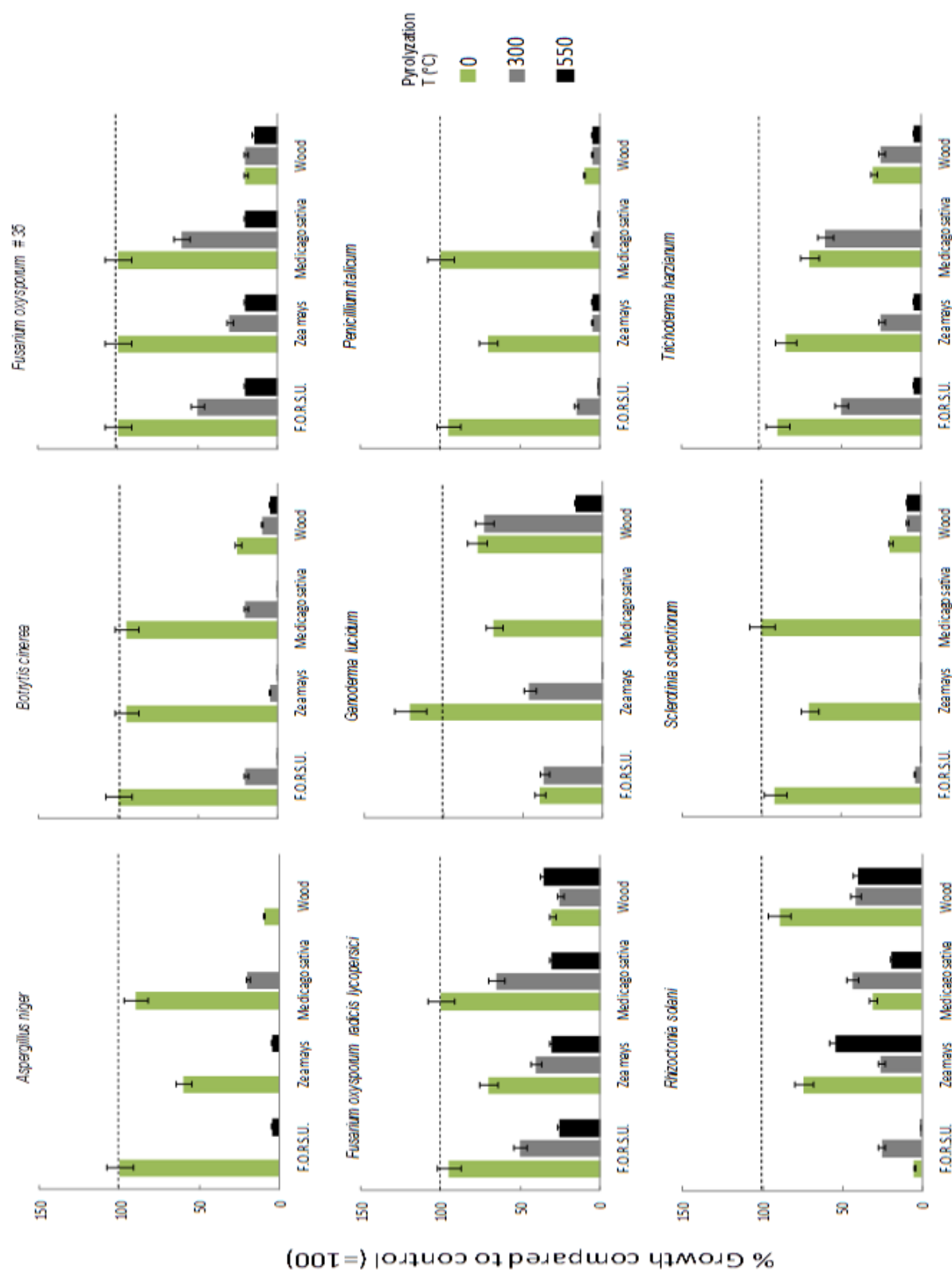
Fig. 3. Seed germination: responses of three plant species exposed to watery extracts (50 g l⁻¹) of organic materials untreated (O °C) or biochar obtained at two different temperatures (300 °C and 550 °C) for 5 hours, roots growth expressed as percentage of unexposed controls. Values are average of 10 replicates.



4.4.3 Biochar effects on fungal growth

In the spore germination and hyphal growth bioassays, the response of microbes to extracts of biochars and organic materials showed similar results for most of the fungi. In particular, seven fungi (*Aspergillus*, *Botrytis*, the two *Fusarium*, *Penicillium*, *Sclerotinia*, *Trichoderma*) grew on untreated F.O.R.S.U., *Zea*, and *Medicago* almost as the control (PDA). Wood showed a remarkable inhibitory effect on all target species except for *Rhizoctonia* and *Ganoderma* (Fig. 4). All biochars produced at 300°C displayed a consistent growth reduction of all fungal species (Fig. 4). In particular, a trend of decreasing growth was progressively confirmed at higher temperatures, with six of the nine fungi completely inhibited on extracts of biochars produced at 550°C. On this biochar species of *Fusarium* and *Rhizoctonia* showed a strong inhibition (Fig. 4).

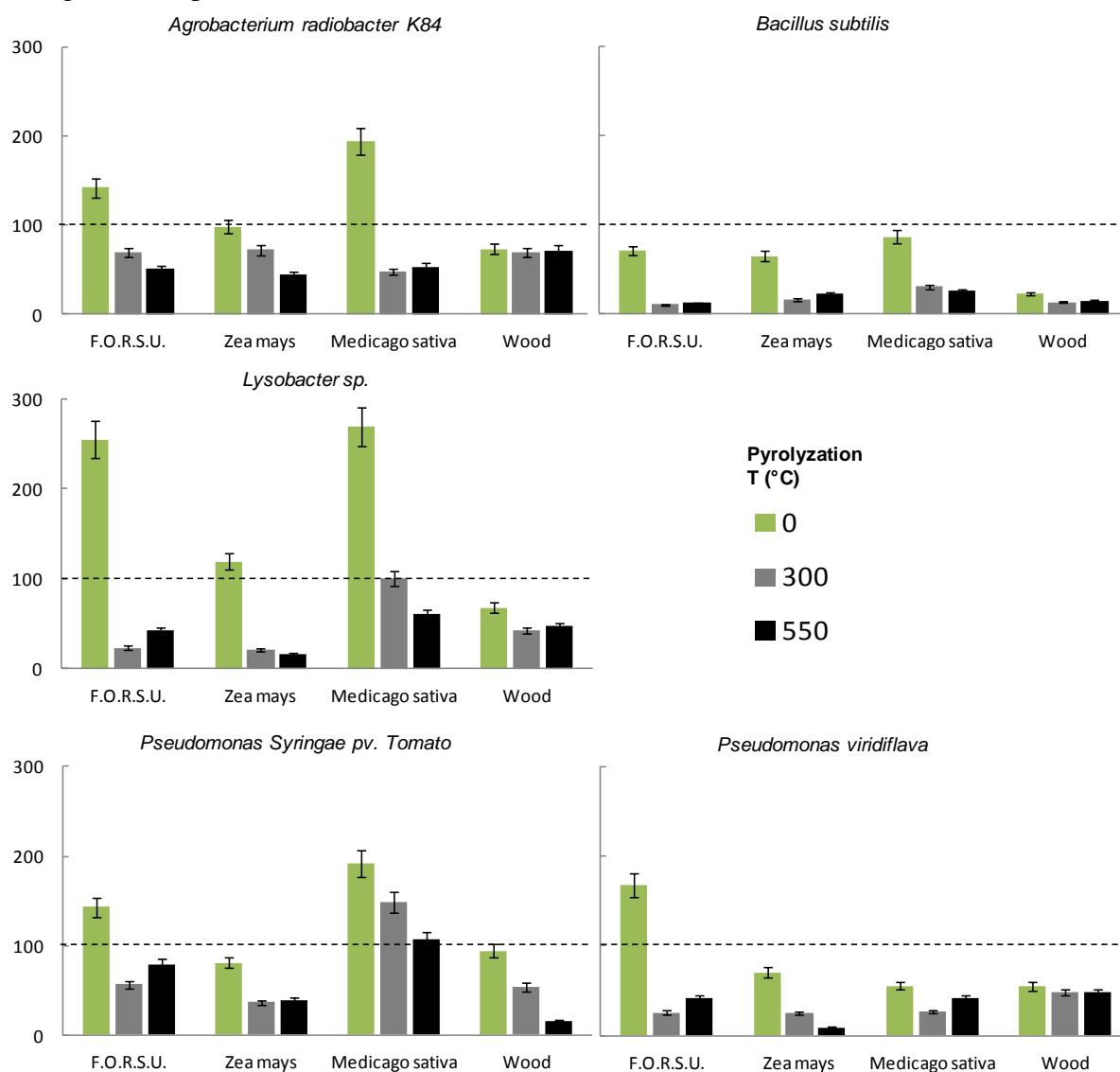
Fig. 4. . Responses of nine fungal species exposed to water extracts (50 g l⁻¹) from organic materials untreated (O °C) or from biochar obtained at two different temperatures (300 °C and 550 °C) for 5 hours. Hyphal growth and spore germination and are expressed as percentage of unexposed controls. Values are average of 10 replicates.



4.4.4 Biochar effects on bacterial growth

Bacterial response to extracts of biochars and organic materials showed a distinct behaviour. F.O.R.S.U. showed a stimulatory effect on untreated material and an inhibitory effect that progressively increased at higher heating temperatures. (Fig. 5). Also untreated *Medicago* had a positive effect on some bacteria, while biochar extracts inhibited bacterial growth. *Zea* and wood feedstocks had a similar result: a microbial growth lower on biochar extracts than on organic material extracts was observed (Fig. 5).

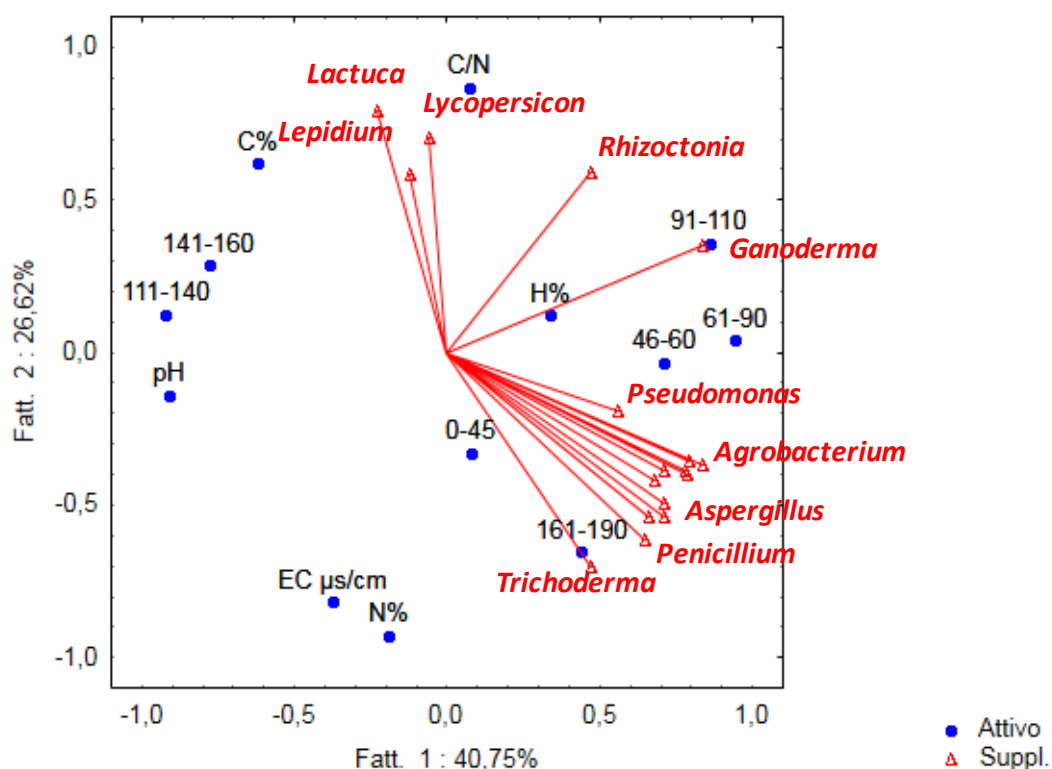
Fig. 5 Responses of five bacterial species exposed to watery extracts (50 g l⁻¹) of organic materials untreated (0 °C) or biochar obtained at two different temperatures (300 °C and 550 °C) for 5 hours, bacterial growth expressed as percentage of unexposed controls. Values are average of 10 replicates.



4.4.5 Organic matter chemistry and target species sensitivity

The ^{13}C -CPMAS NMR data of organic materials were significantly correlated with plant and microbe growth (Table 3). *Lepidium*, *Lactuca* and *Lycopersicon* root growth was positively correlated with (91-160 ppm) spectral regions of ^{13}C -CPMAS NMR derived from the literature, and negatively with carboxyl C regions (161-190 ppm) (Table 3). Moreover, significant positive correlations were found between plant growth with C content and C/N ratio (Table 2).

Fig. 6. . Principal component analysis (PCA) ordination of eight selected ^{13}C NMR spectral regions in 4 organic materials and 8 biochars tested for inhibition of root growth of 3 plants, spore germination of 6 fungi, hyphal growth of 3 fungi and growth of 5 bacteria.



Principal component analysis (PCA) provided a satisfactory ordination of the organic material and biochar chemistry parameters, with the first two eigenvalues accounting for 67.37% (40.75, and 26.62%) of the total variance. In Fig 5 are reported the loading vectors of organic materials and biochar quality parameters (i.e. for each ^{13}C -NMR region, the relative abundance measured on each feedstock sample, while for elemental chemistry values actually recorded in organic materials and biochars, and how they relate to the PC axes), and the factorial scores of the 14 microbes and 3 plants on the bi-dimensional space. The first two

components show the individual organic material sample spreading according to biochemical variations during the pyrolyzation process and the associated effect on plant roots and microbe growth, and the related trajectories of the different species in the multivariate ordination space. Plant growth was positively correlated with relative abundance of labile C and C/N ratio, in addition we observed negative values statistically significant with relative abundance of N and high values of EC (Table 3).

The highest negative correlation was recorded for the microbial growth with ^{13}C NMR H-C-substituted aromatic C regions and relative abundance of labile C and C/N ratio and high values of pH. In addition, we observed positive correlation between microbial growth with carboxylic C region (Table 3). In particular *Aspergillus*, two species of *Fusarium*, *Agrobacterium*, *Lysobacter* and *Pseudomonas syringae* were positively correlated with the amount of N. Instead, *Rhizoctonia* and *Ganoderma* were negatively correlated with the amount of N. The other six species (the fungi *Botritis*, *Trichoderma*, *Penicillium*, *Sclerotinia*, and the bacteria *Bacillus* and *Pseudomonas viridiflava*) did not correlated with N content (Table 3).

Table 2. Synthetic results of 17 two-way ANOVA for two pyrolyzation temperatures and twelve feedstocks. Data refer to degrees of freedom (df) and F statistics for main and interactive effects.

Species	Source	df	F	P-value
<i>Lactuca sativa</i>	Heating temperature (HT)	2	32.13	<0.01
	Feedstocks (F)	3	77.06	<0.01
	HT x F	6	11.20	<0.01
<i>Lepidium sativum</i>	Heating temperature (HT)	2	94.12	<0.01
	Feedstocks (F)	3	160.02	<0.01
	HT x F	6	60.32	<0.01
<i>Lycopersicon esculentum</i>	Heating temperature (HT)	2	124.67	<0.01
	Feedstocks (F)	3	402.48	<0.01
	HT x F	6	57.08	<0.01

<i>Aspergillus niger</i>	Heating temperature (HT)	2	2323.47	<0.01
	Feedstocks (F)	3	331.18	<0.01
	HT x F	6	238.40	<0.01
<i>Botrytis cinerea</i>	Heating temperature (HT)	2	841.51	<0.01
	Feedstocks (F)	3	54.93	<0.01
	HT x F	6	54.68	<0.01
<i>Trichoderma harzianum</i>	Heating temperature (HT)	2	4.94	0.015
	Feedstocks (F)	3	1.01	0.40
	HT x F	6	0.69	0.65
<i>Penicillium italicum</i>	Heating temperature (HT)	2	6.00	0.007
	Feedstocks (F)	3	1.36	0.278
	HT x F	6	0.95	0.477
<i>Fusarium oxysporum</i> #35	Heating temperature (HT)	2	355.06	<0.01
	Feedstocks (F)	3	100.12	<0.01
	HT x F	6	38.94	<0.01
<i>Fusarium oxysporum radialis lycopersici</i>	Heating temperature (HT)	2	6.26	0.006
	Feedstocks (F)	3	3.73	0.02
	HT x F	6	1.64	0.17
<i>Ganoderma lucidum</i>	Heating temperature (HT)	2	4.35	0.02
	Feedstocks (F)	3	1.28	0.30
	HT x F	6	0.95	0.47
<i>Rhizoctonia solani</i>	Heating temperature (HT)	2	0.99	0.38

<i>Sclerotinia sclerotiorum</i>	Feedstocks (F)	3	2.04	0.13
	HT x F	6	0.65	0.68
	Heating temperature (HT)	2	3.38	0.05
<i>Agrobacterium radiobacter K84</i>	Feedstocks (F)	3	0.080	0.96
	HT x F	6	0.98	0.45
	Heating temperature (HT)	2	214.55	<0.01
<i>Bacillus subtilis</i>	Feedstocks (F)	3	18.31	<0.01
	HT x F	6	45.10	<0.01
	Heating temperature (HT)	2	343.57	<0.01
<i>Lysobacter</i>	Feedstocks (F)	3	130.15	<0.01
	HT x F	6	36.38	<0.01
	Heating temperature (HT)	2	0.98	0.38
<i>Pseudomonas syringae pv. tomato</i>	Feedstocks (F)	3	0.62	0.60
	HT x F	6	1.04	0.41
	Heating temperature (HT)	2	2.70	0.08
<i>Pseudomonas viridiflava</i>	Feedstocks (F)	3	6.43	0.002
	HT x F	6	0.96	0.47
	Heating temperature (HT)	2	5.24	0.01
	Feedstocks (F)	3	2.45	0.08
	HT x F	6	1.55	0.20

The performance of all species, bacterial and fungal data, were positively associated with restricted resonance regions at 46-60 ppm, 61-90 ppm and 161-190 ppm and negatively

with the aromatic region at 111-160 ppm (Fig. 4; Fig.5). This was a general pattern with only slight interspecific differences, as in the cases of *Rhizoctonia* and *Ganoderma*, that showed a positive correlation with the resonance region at 91-110 ppm.

Table 3: Linear correlation between microbial and plants growth with biochar and organic materials chemical parameters and ^{13}C -CPMAS NMR spectral regions

	C%	N%	C/N	pH	EC $\mu\text{s}/\text{cm}$	Alkyl C:	Methoxyl and N-alkyl C:	O-alkyl C: 61-90 ppm	di-O-alkyl C: 91-110 ppm	H- & C-sub. aromatic C: 111-140 ppm	O-sub. aromatic C:	Carboxylic C: 161-190 ppm
<i>Lactuca sativa</i>	0,66 (0.019)	-0,62 (0.030)	0,61 (0.035)	0,04	-0,71 (0.009)	-0,06	-0,25 (0.42)	-0,25 (0.44)	0,08	0,24 (0.45)	0,47 (0.119)	-0,64 (0.023)
<i>Lycopersicon esculentum</i>	0,46 (0.19)	-0,62 (0.032)	0,36 (0.24)	-0,14	-0,74 (0.056)	-0,04	-0,15	-0,08	0,23 (0.47)	0,09	0,32 (0.30)	-0,65 (0.020)
<i>Lepidium sativum</i>	0,40 (0.20)	-0,44 (0.15)	0,28 (0.37)	-0,08	-0,56 (0.06)	0,03	-0,26 (0.41)	-0,13	0,16	0,10	0,37 (0.23)	-0,71 (0.009)
<i>Aspergillus niger</i>	-0,62 (0.030)	0,29 (0.036)	-0,33 (0.29)	-0,59 (0.044)	0,13	0,12	0,71 (0.009)	0,66 (0.020)	0,34 (0.28)	-0,68 (0.15)	-0,78 (0.002)	0,78 (0.002)
<i>Botrytis cinerea</i>	-0,66 (0.19)	0,16	-0,26 (0.42)	-0,73 (0.006)	-0,02	0,13	0,74 (0.006)	0,78 (0.002)	0,52 (0.08)	-0,79 (0.002)	-0,82 (0.001)	0,67 (0.017)
<i>Trichoderma harzianum</i>	-0,62 (0.032)	0,20	-0,29 (0.36)	-0,71 (0.008)	-0,04	0,49 (0.10)	0,76 (0.004)	0,65 (0.023)	0,43 (0.159)	-0,88 (0.0001)	-0,68 (0.0149)	0,53 (0.076)
<i>Penicillium italicum</i>	-0,64 (0.026)	0,18	-0,32 (0.31)	-0,68 (0.14)	0,01	0,13	0,72 (0.008)	0,72 (0.007)	0,46 (0.12)	-0,74 (0.005)	-0,79 (0.002)	0,68 (0.015)
<i>Fusarium oxysporum</i> #35	-0,68 (0.014)	0,33 (0.29)	-0,45 (0.14)	-0,60 (0.039)	0,13	0,35 (0.26)	0,67 (0.016)	0,61 (0.035)	0,33 (0.291)	-0,76 (0.0043)	-0,73 (0.006)	0,67 (0.018)
<i>Fusarium oxysporum radicis lycopersici</i>	-0,61 (0.033)	0,41 (0.190)	-0,44 (0.147)	-0,52 (0.083)	0,16	0,42 (0.172)	0,70 (0.011)	0,51 (0.090)	0,19	-0,71 (0.009)	-0,74 (0.005)	0,83 (0.0009)
<i>Ganoderma lucidum</i>	-0,33 (0.294)	-0,45 (0.146)	0,32 (0.311)	-0,88 (0.0001)	-0,56 (0.056)	-0,02	0,61 (0.035)	0,79 (0.002)	0,89 (0.0001)	-0,76 (0.0043)	-0,41 (0.184)	0,06

<i>Rhizoctonia solani</i>	-0,14 (0.008)	-0,72 (0.180)	0,41 (0.246)	-0,36 (0.030)	-0,62 (0.493)	-0,22	-0,02	0,53 (0.073)	0,71 (0.0091)	-0,32 (0.314)	-0,30 (0.351)	-0,35 (0.258)
<i>Sclerotinia sclerotiorum</i>	-0,59 (0.042)	0,17	-0,20	-0,69 (0.012)	-0,01	0,02	0,74 (0.005)	0,74 (0.006)	0,47 (0.123)	-0,71 (0.010)	-0,80 (0.001)	0,76 (0.003)
<i>Agrobacterium radiobacter K84</i>	-0,48 (0.113)	0,26 (0.405)	-0,15	-0,65 (0.021)	-0,01	0,13	0,76 (0.004)	0,58 (0.048)	0,34 (0.273)	-0,66 (0.020)	-0,66 (0.019)	0,88 (0.0001)
<i>Bacillus subtilis</i>	-0,70 (0.011)	0,19	-0,32 (0.304)	-0,47 (0.121)	0,14	-0,01	0,47 (0.126)	0,64 (0.023)	0,43 (0.164)	-0,58 (0.048)	-0,72 (0.007)	0,68 (0.014)
<i>Lysobacter</i>	-0,59 (0.041)	0,35 (0.264)	-0,27 (0.404)	-0,51 (0.091)	0,21	0,16	0,71 (0.009)	0,57 (0.051)	0,26 (0.422)	-0,64 (0.026)	-0,74 (0.005)	0,89 (0.0001)
<i>Pseudomonas syringae pv. tomato</i>	-0,74 (0.005)	0,55 (0.066)	-0,39 (0.211)	-0,26 (0.406)	0,52 (0.082)	0,25 (0.424)	0,42 (0.168)	0,40 (0.193)	0,14	-0,52 (0.081)	-0,54 (0.071)	0,77 (0.003)
<i>Pseudomonas viridiflava</i>	-0,30 (0.343)	0,05	-0,02	-0,52 (0.080)	-0,02	0,00	0,68 (0.015)	0,54 (0.068)	0,30 (0.337)	-0,51 (0.088)	-0,60 (0.038)	0,50 (0.099)

4.5 DISCUSSION

4.5.1. Organic material chemical changes associated with pyrolyzation

The F.O.R.S.U. and *Medicago* feedstocks had higher relative abundance of carboxyl C, di-O-alkyl, O-alkyl C and methoxyl C compared to Wood and Zea. Noteworthy, Wood showed specific features because had the highest relative abundance of the H- and C- substituted aromatic C regions (111-140 ppm) and the lowest of Alkyl C (0-45 ppm).

All organic materials, pyrolyzed at 300°C and 550°C showed dramatic changes as assessed by ¹³C CPMAS NMR analysis compared to the untreated feedstocks. At 300°C a degradation of carboxyl C, di-O-alkyl, O-alkyl C and methoxyl C was observed. At this pyrolyzation temperature, the neo-formation of Alkyl-C and aromatic C compounds was observed.

In the previous studies, a rapid depletion of the O-alkyl C fraction has been reported for both peat (Almendros et al., 2003) and grass residues (Knicker et al., 2005) treated at 350°C for few minutes. Furthermore, at lower temperatures the sensitivity of the O-alkyl C fraction to thermal degradation is inversely related to litter lignin content (Bonanomi et al.,

2016). However, degradation of the O-Alkyl C obtained by pyrolyzation in this study, was higher than that observed in the previous one. This probably happened because the pyrolyzation lasted five hours versus thirty minutes. Moreover all organic materials treated at 300°C showed a significant increase of the alkyl C fraction, mainly related to aliphatic compounds (Fig. 1), which was more evident for F.O.R.S.U. and *Medicago*. Neo-formation of aliphatic compounds for thermally treated soils (Almendros et al., 1998; Knicker et al., 2005) and peat (Almendros et al., 2003) has been reported and related to condensation of water repellent, aromatic polymers. However, also the more stable, paraffin like, alkyl C fractions dramatically decreased at 550°C.

At 300 °C the aromatic and phenolic C fractions (from 111 to 160 ppm), typical of charred plant residues (Knicker, 2007), increased in all biochars. This observation supports the hypothesis that aromatic C compounds do not reflect only a selective, relative enrichment deriving from as the removal of other compounds, but also a thermal neo-formation that likely involves previously dehydrated carbohydrates (González-Pérez et al., 2004). A similar process may drive to a massive production of the aromatic and phenolic fraction that dominate the ^{13}C CMPAS NMR spectra of litter heated at 550°C.

4.5.2. Organic matter preference by plant and microbes

In this study we found that not all undecomposed organic materials caused a severe inhibition of plant root growth. *Medicago* and F.O.R.S.U., displayed an inhibitory effect in agreement with previous studies carried out in both natural and agro-ecosystems, (Dorrepaal et al., 2007; Bonanomi et al., 2011a; Lopez-Iglesias et al., 2014; Meiners, 2014; Mazzoleni et al., 2015), whereas wood and Zea had a stimulating effect on the root growth regardless of the plant test. However, inhibition by organic materials largely decreased for *Medicago* and disappeared for F.O.R.S.U. after pyrolyzation at 300°C-. On the water extracts at 550 ° C the plant growth continued but to a lesser extent compared to 300 ° C.

Previous study showed that the inhibitory effect of root growth by organic materials could be related either to litter N immobilization (Hodge et al., 2000) or to the phytotoxic activity by allelopathic factors (Bonanomi et al., 2011a). According to the nutrient immobilization hypothesis, litter with high C/N ratio, by reducing the available nitrogen, limits root system development. Our results contradict this hypothesis because the inhibitory effect was stronger for species with lower C/N ratios (e.g. F.O.R.S.U. and *Medicago*). This indicates that, at least for the selected species and under laboratory conditions, root growth is

not inhibited by mineral N starvation over organic materials. Probably, other factors are involved in the general inhibitory effects of undecomposed materials, although N limitation may be important in specific ecological conditions, e.g. in presence of litter with high C/N ratios and N poor soils (Michelsen et al., 1999; Bowman et al., 2004). Concerning the alternative hypothesis that organic materials can release allelopathic compounds with harmful effects on root growth (Rice, 1984), we found that *Lepidium*, *Lactuca* and *Lycopersicon* root growth was negatively correlated with the methoxyl C and especially O-alkyl C regions of ^{13}C CPMAS NMR spectra. This result is consistent with a previous study carried out with plant litter decomposed in soil microcosms (Bonanomi et al., 2011a) and indicates that poorly lignified plant materials have inhibitory effects on root proliferation. On the other hand, root growth was positively associated to the two aromatic regions of ^{13}C NMR spectra (Table 2). The extensive correlation analysis applied along the whole ^{13}C NMR spectra also showed a consistent positive association of aromatic C types with root growth. In fact, the entire H- and C-substituted aromatic C region (111-140 ppm) in general was positively correlated to *Lepidium* root growth. Our results, instead, indicate that aromatic and aryl C compounds do not inhibit or, even promote plant growth. A further explanation for the plant growth promoted by biochar can be related to its well-known capability to adsorb and neutralize phytotoxic organic molecules with a limited impact on mineral nutrients (Zackrisson et al., 1996; Hille & den Ouden, 2005). In general, our findings are in agreement with the growing body of literature showing the positive effect of biochar used as amendment in various agricultural systems (Jeffery et al., 2011). This work confirms and validates our previous study (Bonanomi et al., 2016). In addition, further studies are required to clarify the molecular and physiological basis of the positive effects of aromatic and phenolic fractions on plant growth.

In our experiments, fungi and bacteria thrive on most water extracts of organic materials, with growth rates remarkably higher, similar or slightly lower than those recorded for the controls, over rich, standard microbiological substrates (PDA). However, a certain variability exists among feedstocks, with ascomycetes and bacteria preferring fast decomposing, herbaceous, nitrogen-fixing or lignin poor organic material (i.e. *Medicago*, *Zea* and F.O.R.S.U.), rather than highly lignified organic material (Wood). These results are consistent with those by Bonanomi et al., (2011b) and Incerti et al., (2013) reporting a high growth rate of 18 fungal species over several undecomposed leaf litter.

Instead basidiomycetes show a growth rate similar or slightly lower than that recorded for the controls on wood and a less marked growth or an inhibition effect on F.O.R.S.U. *Rhizoctonia*

and *Ganoderma* are reported to be aggressive saprophytes, especially on fresh, herbaceous plant residues, mostly green manure (Rothrock and Kirkpatrick, 1995;). However, their ability to colonize lignin and tannin rich materials is quite limited, likely because of their limited enzymatic arsenal (Sneh et. al., 1996) compared with saprophytic microbes.

In contrast to the higher plants, all tested microbes showed either a steep decline or a complete growth inhibition over biochars at 300°C and 550°C. Such a finding could be explained by the temperature-dependent reduction of litter biochemical quality, which makes the organic materials unsuitable for microbial exploitation. Changes of litter suitability as a substrate for microbial growth could be related to: (i) a decrease of easily degradable C sources; (ii) an accumulation of toxic and/or aromatic organic compounds. In this respect, ^{13}C NMR data clearly show, as litter is progressively heated at increasing temperatures, a sharp decrease of the labile C fraction and a relative increase of aromatic, char typical C compounds. In detail, ^{13}C -CPMAS NMR analysis of peat (Almendros et al., 2003) and grass residues (Knicker et al., 2005) reported a rapid temperature-dependent reduction of carbohydrates (spectral regions at 61-110 ppm, corresponding to di-O-alkyl C and O-alkyl C). This supports the hypothesis that microbial growth inhibition over heated litter is controlled by the availability of easily degradable carbon sources. On the other hand, the possibility exists that newly formed aromatic and phenolic compounds, typical products of charred plant residues, may directly inhibit microbial growth. In conclusion, our findings suggest that the effect of undecomposed organic materials and biochars at different temperatures on microbes can be the result of the balance between the availability of labile organic C sources and the presence of recalcitrant and/or fungitoxic compounds that provide little support or even inhibit microbial growth. Finally, it is noteworthy that the fourteen tested microbes showed a remarkably similar pattern of correlation between growth and organic materials quality, as defined by ^{13}C CPMAS NMR. An opposite response was evidenced by the plants *Lepidium*, *Lactuca* and *Lycopersicon* in terms of both chemical quality and its correlation with root growth. These new results confirm the relationships between the biochemical quality of organic matter and biochars and the multiple effects on different ecosystem trophic levels.

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